c-JUN AND ITS TARGETS IN FIBROSARCOMA AND MELANOMA CELLS

Mari Kielosto

Medicum
Department of Pathology
Faculty of Medicine

Doctoral Programme in Integrative Life Science

and

Faculty of Biological and Environmental Sciences

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Supervisor	Docent Erkki Hölttä, MD, PhD Faculty of Medicine University of Helsinki
Thesis committee	Professor Antti Vaheri, MD, PhD Faculty of Medicine University of Helsinki
	Professor Jim Schröder, PhD Faculty of Biological and Environmental Sciences University of Helsinki
Reviewers	Docent Jarmo Käpylä, PhD Department of Biochemistry University of Turku
	Docent Päivi Koskinen, PhD Department of Biology University of Turku
Opponent	Docent Liisa Nissinen, PhD Department of Dermatology and Venereology University of Turku
Custos	Professor Juha Partanen, PhD Faculty of Biological and Environmental Sciences University of Helsinki

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To my family

ABSTRACT

c-Jun, a member of the AP-1 transcription factor family, is involved in numerous cell activities such as proliferation, differentiation, tissue morphogenesis, tumorigenesis, and apoptosis. c-Jun is a basic leucine zipper (bZIP) transcription factor that can form homodimers and heterodimers with other AP-1 family members. As a dimer, it is able to bind to DNA and regulate transcription of different genes. Numerous extracellular stimuli, such as ultraviolet (UV) radiation, and cellular stimuli, such as reactive oxygen species (ROS), induce signaling cascades leading to phosphorylation and activation of c-Jun. The main kinase phosphorylating c-Jun is c-Jun N-terminal kinase (JNK). c-Jun is constitutively activated in many human cancers, including melanoma, breast, pancreatic, and colorectal cancers, and transformed cell lines, like Ha-*ras* transformed fibroblasts. Thus, better knowledge of the activation of c-Jun and the genes it regulates in cell transformation is needed in the fight against cancer.

The aim of this study was to clarify the role of c-Jun in cell transformation by using fibrosarcoma and melanoma cells as models. In the first part of the study, the significance of phosphorylation and activation of c-Jun in S-adenosylmethionine decarboxylase (AdoMetDC)-, ornithine decarboxylase (ODC)-, and Ha-*ras* oncogene-transformed mouse fibroblasts (Amdc, Odc, and E4 cells, respectively) was examined by exploiting transactivation domain deletion mutant of c-Jun (TAM67) and phosphorylation-deficient c-Jun mutants. Further, the upstream kinases of c-Jun were evaluated by using dominant negative mutants of SEK1 (MKK4) and JNK1 as well as JNK inhibitors. The transformed morphology of the cells was reversed with differing efficacies when transfected with these mutants, most effectively when using TAM67. Due to the highest potency of TAM67, Amdc, Odc, and E4 cells carrying a tetracycline-inducible expression system of TAM67 were then generated (Amdc-, Odc-, and E4- pLRT-TAM67 cell lines). These inducible cell lines provide good, reversibly regulatable models to identify the mechanisms of c-Jun-related transformation. Indeed, expression of TAM67 inhibited cell growth in soft agar and three-dimensional (3D) Matrigel matrix, and, most importantly, tumor formation in *nude* mice.

In the second part of the study, the transformation-relevant genes regulated by c-Jun in Amdc, Odc, and E4 cells were identified by utilizing the above-mentioned cell lines (Amdc-, Odc-, and E4-pLRT-TAM67). After TAM67 induction, the differentially expressed genes in the morphologically normalized cells compared with the transformed cells were identified by Incyte Genomics' cDNA microarray analysis. Relatively few changes were identified,

including those of integrins α6 and β7 (Itgα6 and Itgβ7), which were upregulated in transformed cells, and lysyl oxidase (Lox), which was downregulated. In addition to Lox, also Lox-like-1 and 3 were found to be downregulated in Odc and E4 cells by Affymetrix's microarray and RT-PCR analyses.

In the third and fourth parts of the study, the functional roles of the up- and downregulated genes were examined. Itgα6 was found to pair mainly with Itgβ1 to form integrin α6β1 heterodimer, and function-blocking antibodies against Itgα6 or Itgβ1 inhibited the binding of Amdc cells to laminin and cell invasion in 3D Matrigel. Importantly, similar results were seen with human HT-1080 fibrosarcoma cells. Further, downregulated Lox (pro-LOX) was observed to be involved in the invasion of Odc cells. In addition to fibrosarcoma cells, the expressions of LOX family members were also examined in different human melanoma cell lines, where they were variably expressed. LOXL2 and LOXL3 were upregulated in nearly all melanoma cell lines studied. Upregulated LOX family members and their activities were found to be associated with invasion in melanoma cells, especially when co-cultured with fibroblasts in 3D Matrigel.

In conclusion, we have demonstrated that the transformed phenotype of ODC-, AdoMetDC-, and Ras-transformed mouse fibroblasts is reversibly regulatable by dominant negative mutants of c-Jun and identified Itgα6 and Lox as transformation-relevant target genes of c-Jun. Inactive pro-LOX is suggested to act as a tumor suppressor in these cells. In human melanoma cells, in turn, active LOX and LOXL2 were identified as molecules promoting invasive growth and could offer potential new targets for therapeutic approaches in melanomas.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following four original publications, which are referred to in the text by Roman numerals I-IV:

I Paasinen-Sohns, A., **Kielosto, M.**, Kääriäinen, E., Eloranta, T., Laine, A., Jänne, O.A., Birrer, M.J., and Hölttä E. c-Jun activation-dependent tumorigenic transformation induced paradoxically by overexpression or block of S-Adenosylmethionine Decarboxylase. (2000) *J. Cell. Biol.*151:801-809.

II **Kielosto, M.**, Nummela, P., Katainen, R., Leaner, V., Birrer, M.J., and Hölttä, E. Reversible regulation of the transformed phenotype of Ornithine Decarboxylase- and Ras-overexpressing cells by dominant-negative mutants of c-Jun. (2004) *Cancer Res.* 64:3772-3779.

III **Kielosto, M.***, Nummela, P.* Järvinen, K., Yin, M., and Hölttä, E. Identification of integrins alpha6 and beta7 as c-Jun- and transformation-relevant genes in highly invasive fibrosarcoma cells. (2009) *Int. J. Cancer.* 125:1065-1073.

IV **Kielosto, M.**, Eriksson, J., Nummela, P., Yin, M., and Hölttä, E. Divergent roles of lysyl oxidase family members in ornithine decarboxylase- and RAS-transformed mouse fibroblasts and human melanoma cells. (2018) *Oncotarget.* 9:37733-37752.

*) Equal contribution

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ABBREVIATIONS

AdoMetDC	S-adenosylmethionine decarboxylase
AP-1	activator protein-1
ATF	activating transcription factor
Az	antizyme
AzI	antizyme inhibitor
BAPN	β-aminopropionitrile
bZIP	basic-region leucine zipper
c-Fos	cellular Fos
c-Jun	cellular Jun
Cop1	constitutive photomorphogenesis protein 1
CRE	cAMP-response element
CREB	cAMP-response element-binding protein
dox	doxycycline
DUSP	dual specificity phosphatase
Dz13	DNAzyme 13
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2 (Autotaxin)
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FBLN5	fibulin-5
FBS	fetal bovine serum
FBW7	F-box and WD repeat domain-containing 7
FRA-1/2	FOS-related antigen 1/2
Gapdh	glyceraldehyde-3-phosphate dehydrogenase
HDAC3	histone deacetylase 3
HES	human embryonic skin fibroblast
HMGA1	high-mobility group A1
IκBα	NF-κB inhibitor alpha
JDP	Jun dimerization protein
JNK	c-Jun NH_2-terminal kinase
LOX	lysyl oxidase
LOXL1-4	lysyl oxidase-like 1-4
LOX-PP	lysyl oxidase propeptide
LRF 1	liver regeneration factor 1
MAF	musculoaponeurotic fibrosarcoma
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MARE	Maf recognition element
MDM2	mouse double minute 2 homolog/ E3 ubiquitin-protein ligase Mdm2
MFAP5	microfibrillar-associated protein 5
miR	microRNA
MKP	MAPK phosphatase
MMP	matrix metalloproteinase
NBCS	newborn calf serum
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NPP	nucleotide pyrophosphatase/phosphodiesterase
NRL	neural retina-specific leucine zipper protein

ODC	ornithine decarboxylase
PKC	protein kinase C
ROS	reactive oxygen species
RT-PCR	reverse transcription-PCR
SAPK	stress-activated protein kinase
shRNA	short hairpin RNA
siRNA	small interfering RNA
SUMO	small ubiquitin-like modifier
TAM67	transactivation domain deletion mutant of c-Jun
TPA	12-*O*-tetradecanoyl-phorbol 13-acetate
TRE	TPA-response element
UV	ultraviolet
v-Fos	viral Fos
v-Jun	viral Jun
3D	three-dimensional

INTRODUCTION

Cancer was estimated to cause 9.6 million deaths worldwide in 2018 (World Health Organization, 2018), and it is the second most common reason for deaths in Finland, causing 23% of all deaths in 2017 (Statistics Finland, 2017). Cancer incidence is rising because people are living longer. Cancer development is a multistep process. Epigenetic abnormalities and genetic alterations may result in inappropriate gene regulation, leading to cancer development. Tumor growth and progression have been proposed to depend on several acquired capabilities designated as hallmarks of cancer: sustained proliferative signaling, evasion of growth suppressors, replicative immortality, invasion and metastasis, angiogenesis, and resistance of cell death (reviewed in Hanahan and Weinberg, 2000). In addition to these six core hallmarks of cancer, two enabling characteristics and two emerging hallmarks were added to the list characterizing tumor cells: genomic instability and mutability and tumor-promoting inflammation, and capability to modify cellular metabolism to support neoplastic proliferation and avoidance of immune destruction (reviewed in Hanahan and Weinberg, 2011). Different tumor types acquire these capabilities via distinct mechanisms and at various times during the multistep tumorigenesis. Furthermore, progression of the cancer is associated with a complex interplay between the tumor cells and surrounding non-neoplastic cells and the extracellular matrix.

Tumorigenesis can be seen as a dysfunction of signal transduction networks that regulate molecular communications and cellular processes (reviewed in Sever and Brugge, 2015). The alterations that allow cells to overproliferate and escape the controlling mechanisms of survival and migration may map to a multitude of signaling pathways. One of the affected pathways is the mitogen-activated protein kinase (MAPK) cascade, which is critical in many processes related to malignancy. The mammalian MAPK family consists of extracellular signal-regulated kinase (ERK), p38, and c-Jun NH_2-terminal kinase (JNK) (reviewed in Kyriakis and Avruch, 2012). In our study, we investigated especially the JNK pathway, which leads to activation of the transcription factor c-Jun. The main focus was c-Jun and the genes regulated by c-Jun in the malignant cell transformation. c-Jun is one of the members of the activator protein-1 (AP-1) transcription factor complex. It is involved in many cellular processes such as proliferation, apoptosis, differentiation, survival, tumorigenesis, and tissue morphogenesis (reviewed in

Meng and Xia, 2011). In line with this, activated c-Jun plays a role in carcinogenesis and cancer progression (reviewed in Weiss and Bohmann, 2004).

To study the role of c-Jun in cell transformation, we used ornithine decarboxylase (ODC)-transformed mouse fibroblast cells (Auvinen et al., 1992) and S-adenosylmethionine decarboxylase (AdoMetDC)-transformed fibroblasts. ODC and AdoMetDC are the key regulatory enzymes in the biosynthesis of polyamines (reviewed in Miller-Fleming et al., 2015). The polyamines putrescine, spermidine, and spermine are essential for cell proliferation and are involved in cell transformation. In addition, Ha-*ras*^{val12}-transformed fibroblasts were used in the studies. RAS protein family members (HRAS, NRAS, and KRAS) belong to small GTPases, which are attached to the cell membrane and transmit signals within cells. They are mutated in one-third of human cancers (reviewed in Li et al., 2018; Baines et al., 2011).

REVIEW OF LITERATURE

1. MAPK SIGNAL TRANSDUCTION PATHWAY

Cells need to respond to a diverse, complex, and changing set of signals. Changes in protein expression level, localization, activity, and protein-protein interactions are important in signal transduction, enabling cells to react highly specifically to circumstantial changes and vary effectively the response (reviewed in Lee and Yaffe, 2016). Extracellular signals activate the cell surface receptors, e.g. different integrins (reviewed in Rathinam and Alahari, 2010), which transduce signals across the plasma membrane into the cytoplasm. A complex network of signal transducing proteins in the cytoplasm then processes the signals and transduces them into the nucleus, where activated transcription factors regulate the expression of different genes, which in turn are responsible for the different cellular responses. There are numerous different pathways mediating signals in the cells, including the MAPK pathway (reviewed in Weston and Davis, 2007). Transmission signals via the MAPK pathway are usually initiated by activation of small G-proteins, like RAS, followed by activation of a sequential set of protein kinases (reviewed in Shaul and Seger, 2007). In cancer pathogenesis, these signaling cascades do not function properly, leading to abnormal cell proliferation and the potential to invade other parts of the body.

1.1 MAPKs

MAPKs are activated by extracellular and intracellular stimuli involving peptide growth factors, cytokines, hormones, and cellular stress. MAPKs include JNK, ERK, and high osmolarity glycerol response kinase (p38), which are regulated spatio-temporally within cells (reviewed in Atay and Skotheim, 2017; Tomida, 2015). All of these signaling pathways consist of at least three components (see Figure 1): a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK, where MAPKKKs phosphorylate and activate MAPKKs, and MAPKKs in turn phosphorylate and activate MAPKs (reviewed in Dhanasekaran and Johnson, 2007). There are at least 20 MAPKKKs, 7 MAPKKs and 11 MAPKs. Activated MAPKs generally detach from the scaffold and translocate to the nucleus. MAPKs have many different substrates, including predominantly transcription factors, which regulate genes involved in cell proliferation, differentiation, survival, and death. Different MAPKs can activate an overlapping set of transcription factors.

Abnormal MAPK signaling has been implicated in human malignancies. Thus, the MAPK pathways need to be tightly regulated. The MAPK phosphatases (MKPs), also known as dual specificity phosphatases (DUSPs), are a family of proteins functioning as major negative regulators of MAPKs. Dephosphorylation of threonine and/or tyrosine residues within the Thr-X-Tyr motif located in the MAPK activation loop inactivates MAPKs. Further, the MKPs/DUSPs have also been implicated in the development of cancers (reviewed in Low and Zhang, 2016; Kidger and Keyse, 2016).

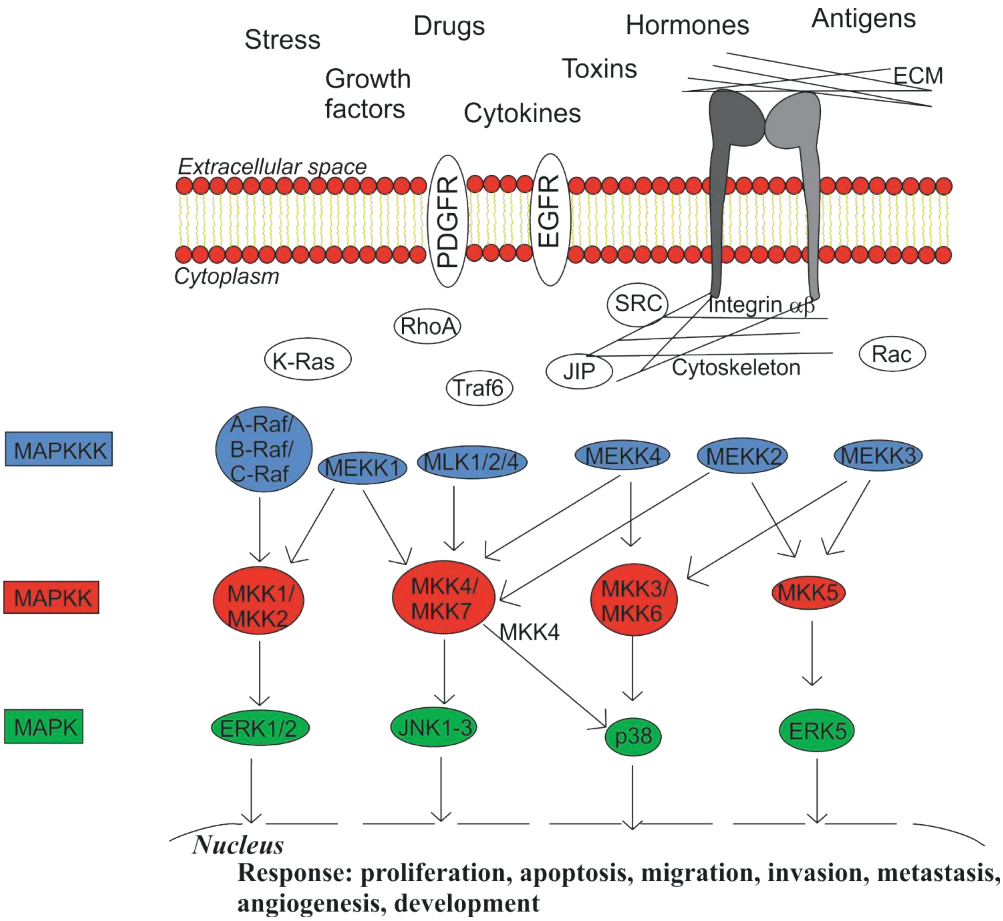


Figure 1. Simplified model of the MAPK signaling network (modified from reviews of Dhanasekaran and Johnson, 2007; Johnson, 2011; Tomida, 2015). The molecules and factors marked in the extracellular space act as upstream activators of the MAPK pathway.

1.1.1 JNKs

JNKs are known as stress-activated protein kinases (SAPKs), and they belong to the MAPK superfamily. Three JNK genes, *Jnk1*, *Jnk2*, and *Jnk3*, are known, but due to alternative splicing there are up to 10 different protein products. While *jnk1* and *jnk2* genes are expressed ubiquitously in all tissues, *jnk3* expression is restricted primarily to the brain, heart, and testes (reviewed in Weston and Davis, 2007; Bogoyevitch and Kobe, 2006; Davis, 2000).

The JNK family members regulate a diverse set of cellular processes, including cell proliferation, differentiation, migration, inflammation, and apoptosis. JNKs are activated by extracellular stimuli caused by stress (UV irradiation, hyperosmolarity, heat shock), but also by intracellular stimuli, such as endoplasmic reticulum (ER) stress, which is caused by the disruption of protein processing and folding within the ER (Win et al., 2014). Furthermore, several growth factors, proinflammatory cytokines (TNF-α, IL-1β), and Toll-like receptor ligands from invading pathogens lead to JNK activation.

The JNK pathway involves the activation of various small G proteins and the engagement of adaptor proteins, followed by activation of a protein kinase cascade, comprising various members of the MAPKKK family (reviewed in Sehgal and Ram, 2013). Finally, JNK is activated by dual phosphorylation performed by the MAPK kinases MKK4 and MKK7 on specific threonine and tyrosine residues in a typical Thr-X-Tyr motif. Activated JNKs can then phosphorylate their substrates in different locations (Tournier et al., 1997; Derijard et al., 1995).

The number of known JNK substrates is close to 100 (reviewed in Bogoyevitch and Kobe, 2006; Zeke et al., 2016). They are predominantly nuclear, such as transcription factors and hormone receptors, but also cytoplasmic proteins, cell membrane receptors, and mitochondrial protein substrates exist. The nuclear translocation of JNKs is a nuclear translocation sequence (NTS)-independent process, mediated by distinct β-like importins (reviewed in Flores et al., 2019). The proto-oncogenic transcription factor c-Jun was the first JNK substrate to be known, thus giving JNKs their name c-Jun N-terminal kinases. JNKs can phosphorylate and activate c-Jun on serines 63 and 73 as the major phosphorylation sites, but also on threonines 91 and 93. In addition to c-Jun, JNKs can phosphorylate and activate other AP-1 family members such as JunB, JunD, and activating transcription factor 2 (ATF2) (reviewed in Bogoyevitch and Kobe, 2006).

JNK signaling has been linked to several pathological conditions such as neurodegenerative diseases, autoimmune diseases, diabetes, asthma, cardiac hypertrophy, and cancer (reviewed in Sabapathy, 2012; Kumar et al., 2015; Cui et al., 2007; Koch et al., 2015). JNKs are thought to have an oncosuppressive role in cancer by mediating apoptosis, but many studies have also implicated them, especially JNK1, in malignant transformation and tumor growth (reviewed in Liu and Lin, 2005; Gkouveris and Nikitakis, 2017; Tournier, 2013; Das et al., 2011). However, also evidence for a predominant role for JNK2 in Ras-induced transformation has been presented (Nielsen et al., 2007). Moreover, JNKs have been shown to be involved in all steps of the metastatic cascade, starting from the promotion of epithelial-to-mesenchymal transition in tumor cells to promotion of proliferation of seeded tumor cells and their surveillance at the metastatic site (reviewed in Ebelt et al., 2013). The diversity of JNK upstream and downstream signaling may lead to contradictory functions of JNK in cancer. In addition, JNK1 and JNK2 have been shown to have discrete or even opposite functions, e.g. JNK1 phosphorylates c-Jun, leading to cell proliferation, while JNK2 reduces c-Jun stability, leading to decreased proliferation (Sabapathy et al., 2004).

Improved understanding of the complexity of JNK signaling can potentially lead to development of novel therapeutic strategies for cancer and other diseases (reviewed in Cui et al., 2007; Kumar et al., 2015; Koch et al., 2015; Xu, and Hu, 2020). For example, anti-cancer compounds that induce severe ROS accumulation, causing activation of JNK-mitochondrial and ER stress pathways and leading to apoptosis of cancer cells, have potential as clinical therapeutic agents (Zou et al., 2015; Che et al., 2017).

1.1.2 ERKs

ERKs, other members of the MAPK superfamily, include ERK1 and ERK2. They are ubiquitous regulators of multiple cellular processes, including proliferation, differentiation, development, cell survival, transformation, and, under some conditions, apoptosis. Similarly to JNKs, also ERKs have been found to be involved in both oncogenesis and tumor suppression (reviewed in Deschenes-Simard et al., 2014). Ras/Raf/MEK/ERK pathway has also been shown to be activated in many cancer types, such as melanoma and colorectal cancer, and ERK inhibitors and other therapeutic agents have been developed (reviewed in McCubrey et al., 2007; Burotto et al., 2014; Savoia et al., 2019; Degirmenci et al., 2020).

ERKs are activated by growth factors and mitogens through the Ras/Raf/MEK/ERK signaling cascade. Ras is a small GTPase, which is mutated in up to 30% of human cancers. Protein kinase Raf, which is also frequently mutated in cancer, is one of the downstream effectors

recruited by Ras. Raf dimers then phosphorylate the dual-specificity kinases MEK (MAPK/ERK kinase), which in turn activate ERK through dual phosphorylation of its regulatory tyrosine and threonine residues (reviewed in Dorard et al., 2017). Activated ERKs can phosphorylate large numbers of substrates, which are localized in the cytoplasm or nucleus. These substrates include signal transduction protein kinases like BRAF, transcription factors such as Elk1, Ets1/2, and MYC, and many of the AP-1 family members like c-Jun, JUNB, JUND, FOS, and ATF2 (http://sys-bio.net/erk_targets/targets_all.html; reviewed in Unal et al., 2017).

1.1.3 p38

p38 MAPK is known as stress-activated MAPK, being responsive to cellular stress and cytokines. Four genes encoding p38 MAPKs are *MAPK14*, encoding p38α, *MAPK11*, encoding p38β, *MAPK12*, encoding p38γ, and *MAPK13*, encoding p38δ. p38α is highly abundant in most cell types, the others having more restricted expression. A specific inhibitor is available for p38α, the thus far best characterized member of the p38 MAPK family (reviewed in Igea and Nebreda, 2015). In addition to having different tissue-specific expression patterns, the p38 family members differ by their regulation of upstream stimuli, selectivity for upstream regulatory kinases and phosphatases, sensitivity to chemical inhibitors and different downstream targets (reviewed in Roux and Blenis, 2004).

Upstream kinases MKK3 and MKK6, and sometimes MKK4, activate p38 MAPK by dual phosphorylation (Derijard et al., 1995), and activated p38 MAPKs in turn are known to regulate by phosphorylation more than 100 proteins. Half of these are transcription factors, including members of AP-1 family: ATF2, c-Fos, c-Jun, and MafA (Trempolec et al., 2013). The rest of the substrates comprise protein kinases and phosphatases, cell cycle and apoptosis regulators, growth factor receptors, and cytoskeletal proteins (Trempolec et al., 2013).

p38 signaling plays an important role in immune response and regulation of cell survival and differentiation. Furthermore, it is involved in different human diseases such as inflammation, cardiovascular dysfunction, Alzheimer's disease, and cancer (reviewed in Cuenda and Rousseau, 2007). Like other MAP kinases, p38 has also both tumor-suppressive and oncogenic functions (reviewed in Hui et al., 2007; Igea and Nebreda, 2015; Bulavin and Fornace, 2004). The role of p38α MAPK signaling in cancer is shown to be cell- and tumor-type dependent (reviewed in Gupta and Nebreda, 2015). For instance, p38α/ATF2 expression plays a crucial role in the malignant phenotype of ovarian tumor cells (Song et al., 2017) and upregulation of

p38 activity accelerates proliferation and migration of breast cancer cells (Huth et al., 2017). However, p38 was found to be significantly less active in human hepatocellular carcinoma tissue than in adjacent non-neoplastic tissue (Iyoda et al., 2003). Interestingly, it has also been shown that p38α has a dual function in colon cancer: suppressing inflammation-associated epithelial damage and tumorigenesis, but promoting proliferation and survival of tumor cells (Gupta et al., 2014).

2. AP-1 TRANSCRIPTION FACTOR

AP-1 is a dimeric transcription factor consisting of members of the JUN (c-Jun, JUNB, and JUND), FOS (c-FOS, FOSB, and FOS-related antigens Fra-1 and Fra-2), ATF (ATF1, ATF2, liver regeneration factor 1 LRF1/ATF3, B-ATF, Jun dimerization proteins JDP1 and JDP2, cAMP-response element-binding protein CREB, cAMP-response element modulator CREM) and musculo-aponeurotic fibrosarcoma MAF (c-Maf, MafB, MafA, MafG/F/K, and neural retina-specific leucine zipper protein Nrl) protein subfamilies. JUN and FOS of these subfamilies constitute the major AP-1 proteins. Common to all of these proteins is the leucine-zipper domain, which is essential for dimerization, and a basic domain required for DNA binding. Dimerization brings together the basic regions, which then interact with specific sequences of DNA. In addition, MAF transcription factors have been thought to belong to the AP-1 superfamily because they share structural similarities like the basic leucine-zipper (bZIP) regions. Their functions are, however, distinctly diversified when compared with the other AP-1 proteins (reviewed in Katsuoka and Yamamoto, 2016).

Due to the multiple dimerization partners, AP-1 proteins can form many combinations of homo- and heterodimers, which in turn determine the gene to be regulated (Bakiri et al., 2002; reviewed in Bejjani et al., 2019). They regulate genes involved in many cellular processes such as cell proliferation, inflammation, differentiation, apoptosis, angiogenesis, migration, and invasion. AP-1 proteins are mainly considered to be oncogenic, but some of them have been shown to have tumor suppressor activity as well. This may depend on the antagonistic activity of different JUN proteins and also the type, stage, and genetic background of tumors (Table 1) (reviewed in Shaulian, 2010; Eferl and Wagner, 2003).

Table 1. Roles of Jun family members in cancer are context-dependent (reviewed in Shaulian, 2010; Eferl and Wagner, 2003).

JUN FAMILY MEMBER		ROLE IN CANCER
c-Jun	Oncogene	enhances cell proliferation, migration, invasion, and angiogenesis and suppresses apoptosis
	Tumor suppressor	induces apoptosis
JunB	Oncogene	migration, invasion, metastasis
	Tumor suppressor	inhibits cellular proliferation and transformation
JunD	Oncogene	induces proliferation, inhibits apoptosis
	Tumor suppressor	downregulates cell growth in response to Ras signal transduction

2.1 c-JUN

The cellular Jun (c-Jun) is a dominant component of AP-1 complexes in many cell lines (Angel, Allegretto et al., 1988; Bos et al., 1988; reviewed in Bohmann et al., 1987). Jun was first characterized as a viral oncoprotein, v-Jun, derived from avian sarcoma virus 17 (ASV17), which causes progressive fibrosarcoma in chicken and transforms chick embryo fibroblasts (Maki et al., 1987). v-Jun differs from c-Jun by a 27-amino acid deletion near the N-terminus (delta deletion), where the binding domain of JNK is situated, and three amino acid substitutions in the C-terminal half (Nishimura and Vogt, 1988). One of these amino acid substitutions of v-Jun is serine-243, which is mutated to phenylalanine. Because of this substitution, glycogen synthase kinase 3 (GSK3) is not able to mark it by phosphorylation, which allows v-Jun to escape recognition and destruction of Fbw7 (F-box and WD repeat domain-containing 7) ubiquitin ligase complex (Wei et al., 2005). However, c-Jun, as a single oncoprotein, is also able to transform rat fibroblast cell line Rat1a on its own, and in cooperation with activated *ras* gene, it can transform primary rat embryo cells (Schutte et al., 1989). In addition, c-Jun is able to act in synergy with oncogenic Ras to transform normal epidermal cells into malignant ones (Jin, J. Y. et al., 2011).

2.1.1 Structure of c-Jun

The human *JUN* gene is located on chromosome 1 at region p31-32 (Haluska et al., 1988; Hattori et al., 1988), whereas murine *Jun* is on chromosome 4, subregion C5-C7 (Mattei et al., 1990). Cloning of the *c-jun* gene surprisingly showed it to be intronless and to have an atypical TATA box (Hattori et al., 1988; Nishimura and Vogt, 1988). The promoter region of the *c-jun*

gene contains a c-Jun/AP-1 binding site, so c-Jun mediates positive autoregulation of its own gene product (Angel, Hattori et al., 1988).

c-Jun is a 39 kDa nuclear phosphoprotein comprising an N-terminal transcriptional activation domain and a C-terminal bZIP domain, which in turn consists of a basic DNA binding domain, followed by an α-helical leucine zipper dimerization domain (Figure 2) (reviewed in Vogt and Morgan, 1990). The locations of functional domains and phosphorylation sites in c-Jun are shown in Figure 2. In the proximity of the N-terminus of c-Jun is the delta domain, to which the MAP kinase JNK binds. JNK then activates c-Jun by phosphorylating its transactivation domain, on serines 63/73 (S63/73), which are the major sites of phosphorylation. Under some circumstances, also threonines 91/93 (T91/93) can be phosphorylated (reviewed in Dunn et al., 2002). Negative regulatory phosphorylation sites of c-Jun are located proximal to the DNA-binding domain on threonine 239 and serines 243 and 249 in the C-terminus. They are phosphorylated by GSK3 (Boyle et al., 1991; Morton et al., 2003). Various stimuli can decrease the C-terminal domain phosphorylation, thereby increasing the DNA binding affinities of c-Jun.

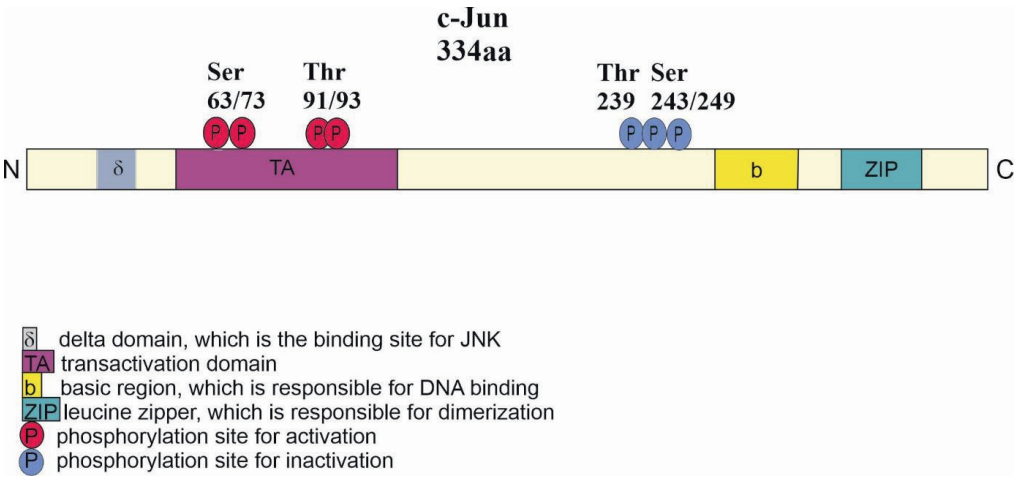


Figure 2. Structure of c-Jun. JNK binding site in the delta domain and JNK phosphorylation sites in the transactivation domain are located in the N-terminus. The C-terminus contains a basic region for DNA binding, leucine zipper for dimerization, and phosphorylation sites for negative regulation.

c-Jun can dimerize with different partners, thus recognizing different sequence elements in the promoters and enhancers of target genes (reviewed in Bejjani et al., 2019). c-Jun, which forms homodimers or dimerizes with JunB, JunD, c-Fos, FosB, FRA1, or FRA2, prefers binding to the TPA-response element (TRE) 5'-TGACTCA-3'. It is so named because it is strongly

induced by the tumor promoter 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA). Heterodimers with c-Fos are more stable than c-Jun homodimers and have a higher affinity for the DNA target sequence. When c-Jun dimerizes with different ATF proteins, it in turn preferentially binds to cAMP-response element (CRE) 5'-TGACGTCA-3' (reviewed in Eferl and Wagner, 2003). Interestingly, it has been found that c-Jun/c-Fos heterodimers can bind methylated promoters when the gene is repressed and can reverse the epigenetic silencing and induce expression of that gene (Gustems et al., 2014).

2.1.2 Activation and regulation of c-Jun

c-JUN is an immediate early gene activated by proinflammatory cytokines, genotoxic stress, ROS, UV radiation, hormones, and growth factors. The activity of c-Jun protein is stimulated by signaling pathways through MAPKs: JNK, ERK, and p38 families. c-Jun N-terminal phosphorylation at serines 63 and 73 and threonine 91 or 93 or both in its transactivation domain increases transcription of c-Jun target genes, including the *c-jun* gene itself. Indeed, c-Jun transcription is directly stimulated by its own gene, creating a positive regulatory loop (Angel, Hattori et al., 1988). In many human cancers, overexpression of c-Jun is the result of upstream oncogene activation, but also amplifications of the c-Jun locus have been observed in undifferentiated and aggressive human sarcomas (Mariani et al., 2007).

c-Jun activity can be regulated by different mechanisms, including transcription, post-translational modification, dimerization of partners, and interaction with accessory proteins. Depending on the regulation mechanisms, a given AP-1 factor can regulate a specific target gene positively or negatively. In the absence of JNK signaling, a repressor complex containing histone deacetylase 3 (HDAC3) interacts with the N-terminal region of c-Jun and inhibits it. Phosphorylation of c-Jun by JNK causes dissociation of the HDAC3 complex and relieves suppression of the transcriptional activity of c-Jun (Weiss et al., 2003).

c-Jun can further be regulated by ubiquitylation and proteasomal degradation. In the G1/S transition, c-Jun rapidly accumulates when quiescent cells are stimulated to proceed in the cell cycle. If the cells return to quiescence, c-Jun is again cleared by ubiquitin-mediated proteolysis. Thus far, three ubiquitin ligases have been reported to trigger ubiquitylation and degradation of c-Jun. One of them is Itch, whose E3 ligase activity needs to be activated by JNKs (Gao, M. et al., 2004). The second ligase is the ubiquiting E3 ligase, Fbw7, which mediates degradation depending on C-terminal phosphorylation of c-Jun by GSK3β (Wei et al., 2005). Previous studies have also shown that adaptor protein Rack1 can enhance oncogenic c-Jun stability by binding with non-phosphorylated N-terminal c-Jun along with Fbw7 to form a complex. When

c-Jun is then phosphorylated by JNKs or other kinases, the Rack1-Fbw7 complex is released from c-Jun, leading to protection of c-Jun from degradation (Zhang, J. et al., 2012). The third ubiquitin ligase is the E3 ubiquitin ligase constitutive photomorphogenesis protein 1 (Cop1), which functions as a tumor suppressor, antagonizing c-Jun oncogenic activity. Thus the loss of Cop1 may be one mechanism leading to c-Jun upregulation in human cancers (Migliorini et al., 2011).

2.1.3 Putative target genes of c-Jun and their functions in tumorigenesis

Oncogene activation is often an early step in neoplastic progression. Aberrant activation of c-Jun is known to be critical in regulation of a complex program of gene expressions involved in the different aspects of tumorigenesis. Figure 3 shows some of the putative genes up- and downregulated by c-Jun during tumorigenesis (Sioletic et al., 2014; reviewed in Eferl and Wagner, 2003). However, previously published data of the genes directly regulated by c-Jun in cancer cells did not include, for example, the genes *cyclin D1* and *p53* (Schummer et al., 2016). This may indicate that some earlier published cancer-related and putative c-Jun regulated genes are probably regulated indirectly by c-Jun.

There are three steps in carcinogenesis: initiation, promotion, and progression, the latter two of which require activated c-Jun. Increased expression and constitutive activation of c-Jun have been detected in multiple human cancers, one of them being malignant melanoma, which is the most aggressive skin tumor (reviewed in Kappelmann et al., 2014). Recently, 44 genes were reported to be directly regulated by c-Jun in cancer cell lines, and six of them were analyzed in more detail in melanoma cells (Schummer et al., 2016). Furthermore, overexpression of c-Jun is detectable in human pancreatic (Tessari et al., 1999), breast (Gee et al., 2000), colorectal (reviewed in Ashida et al., 2005), and squamous cell carcinoma (Jin, J. Y. et al., 2011) as well as in mouse lung tumorigenesis (Tichelaar et al., 2010), suggesting an important role of c-Jun in tumorigenesis.

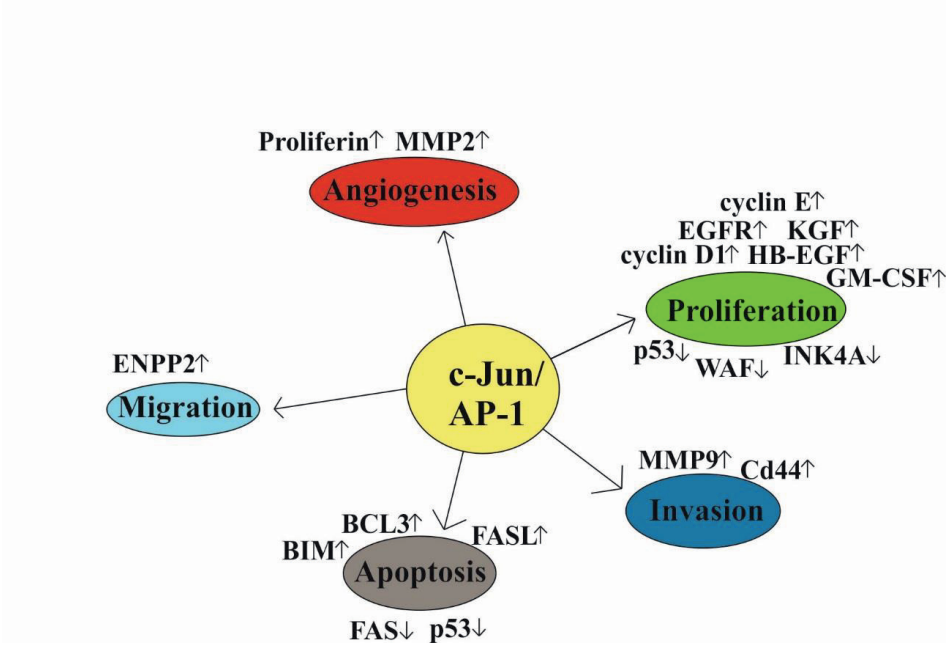


Figure 3. Schematic diagram of putative genes up- or downregulated directly or indirectly by c-Jun during tumorigenesis (modified from Sioletic et al., 2014; Zhang, G. et al., 2006; reviewed in Eferl and Wagner, 2003). EGFR: epidermal growth factor receptor; FASL: FAS ligand; GM-CSF: granulocyte-macrophage colony-stimulating factor; HB-EGF: heparin-binding epidermal growth factor; MMP: matrix metalloproteinase; ENPP2: ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin).

2.1.3.1 c-Jun in cell proliferation

Thus far, most of the aberrantly directly or indirectly regulated target genes of c-Jun have been found to be involved in cell proliferation. c-Jun is indeed one of the regulators of the G1/S transition in the cell cycle. The genes that are positive regulators of cell cycle progression, such as *cyclin D1* and *cyclin A*, are induced by the activated c-Jun-containing AP-1 complex (Bakiri et al., 2000; Katabami et al., 2005). Hennigan and Stambrook (2001) have expressed transactivation domain deletion mutant of c-Jun, TAM67, in human fibrosarcoma cells (HT1080), which led to inactivation of cyclin D1:cdk4/6 and cyclin E:cdk2 complexes and arrested cells in G1, showing c-Jun to upregulate cyclin D1 and cyclin E in fibrosarcoma cells. In the normally regulated cell cycle, c-Jun and junB phosphorylation states and quantities have been shown to vary at the M-G_1 transition (Bakiri et al., 2000). Phosphorylation of JunB leads to lower JunB protein levels in mitotic and early G_1 cells, while N-terminal phosphorylation of c-Jun increases the transactivational potential of c-Jun. JunB represses and c-Jun activates the

cyclin D1 promoter, which is needed for progression through the G_1 phase of the cell cycle. Further, the tumor suppressor BLU (β-catenin in lung cancer) has been reported to inhibit phosphorylation of c-Jun and to lead to downregulation of cyclin D1 promoter activity and cell cycle arrest at G1 phase (Zhang X Ph et al., 2012).

Negative regulators of cell cycle progression, such as tumor suppressor p53 and its target gene CDK inhibitor *p21* and the cyclin-dependent kinase inhibitor INK4A, are, in turn, repressed by c-Jun (Schreiber et al., 1999; reviewed in Kollmann et al., 2011). In line with this, Maritz et al. (2011) found that in cervical cancer, TAM67 inhibits cell proliferation and increases the expression of cell cycle regulatory protein p21, which appeared to be the key player in growth arrest induced by TAM67.

2.1.3.2 c-Jun and apoptosis

Apoptosis is a process of programmed cell death to eliminate unwanted cells from the organism. c-Jun has been shown to either inhibit (Katiyar et al., 2010) or induce (Bossy-Wetzel et al., 1997) cellular apoptosis depending on its expression level, the cell type, and the context of other regulatory influences. Previously, Ferraris *et al.* (2012) showed that under stress, the nucleolar apoptosis-antagonizing transcription factor (AATF) is required as a co-factor for c-Jun-mediated apoptosis. In liver tumors, c-Jun prevents apoptosis by antagonizing the activity of p53 (Eferl et al., 2003).

2.1.3.3 c-Jun and migration

Autotaxin (ENPP2), a secreted nucleotide pyrophosphatase/phosphodiesterase (NPP), first identified as a motility-stimulating factor in melanoma cells (Stracke et al., 1992), has been identified as a special target of v-Jun in v-Jun-transformed chicken embryo fibroblasts (Black et al., 2004). Also, autotaxin/ENPP2 has been found to be a target of c-Jun in human soft tissue sarcomas (Sioletic et al., 2014). Based on these findings, current research is focused on autotaxin as a possible pharmacological target in melanoma (reviewed in Jankowski, 2011).

2.1.3.4 c-Jun and invasion

Cellular invasion takes place in normal biological processes such as development, immune response, and wound healing. In normal cells, invasion is tightly regulated, but overexpressed growth factors and constitutively activated oncogenes are able to directly induce cell motility and invasion. In tumorigenic invasion, the cells gain the ability to move through the basement membrane and three-dimensional space within host tissues. Finally, in metastasis the tumor cells gain access to vascular or lymphatic systems and spread to distant sites to grow. Metastasis is the most life-threatening stage of cancer.

AP-1 activation regulates invasion in metastatic human tumors by activating or repressing the genes involved in invasion (reviewed in Ozanne et al., 2007; Ozanne et al., 2000). For example, endogenous c-Jun can enhance mammary epithelial tumor cellular migration and invasion (Jiao et al., 2010). Transforming growth factor beta (TGF-β), a multifunctional cytokine, has been shown to play a bifunctional role in tumorigenesis and cellular migration. Janowski *et al.* (2011) have shown that TGF-β-induced calcium signaling and migration are dependent on c-Jun. In their study, the authors used floxed c-Jun transgenic mice and compared the *c-jun* wild type with the conditional *c-jun* knockout cells. According to their results, TGF-β induced cell migration, accompanied by a rise in nuclear calcium, which required an intact c-jun/IP_3 signaling pathway.

AP-1 has further been implicated in the regulation of genes involved in matrix remodeling. Degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs) is a crucial step in tumor invasion and metastasis (reviewed in Westermarck and Kahari, 1999). For example, the promoter of collagenase/MMP-1 gene contains an AP-1 binding site. The signals leading to MMP expression have been found to be regulated by EGFR through MAPK and AP-1 pathways (Kajanne et al., 2007). Phosphorylated c-Jun and Fra-1 have further been shown to bind to the AP-1 binding site of the *MMP-1* promoter in 143B osteosarcoma cells, leading to MMP-1 expression and cell invasion (Kimura et al., 2011). Furthermore, also the type IV collagenase, MMP-9, contains an AP-1 binding site in its promoter and c-Jun is one of its regulators. Park *et al.* (2014) have found that the small leucine zipper protein (sLZIP) induces c-Jun and MMP-9 expression in cervical cancer cells, resulting in cell migration and invasion. sLZIP belongs to basic leucine zipper transcription factors of the CRE/ATF gene family. In addition, c-Jun is also involved in regulation of other proteinases, e.g. cathepsin L, degrading the extracellular matrix (Ravanko et al., 2004). Besides invasion and metastasis, MMPs are also capable of promoting angiogenesis.

2.1.3.5 c-Jun and angiogenesis

The activation of c-Jun is also linked to angiogenesis. Angiogenesis is a complex and multilevel process where new blood vessels form from pre-existing vasculature. Under normal conditions, angiogenesis takes place during wound healing and reproduction. Interestingly, Zhang *et al.* (2004) used DNAzyme targeting c-Jun mRNA (Dz13) and showed that the human endothelial cells could no longer form new blood vessels *in vitro* or *in vivo.* Indeed, endothelial cell proliferation, migration, chemoinvasion, and tubule formation was blocked by Dz13. DNAzyme inhibition of c-Jun further suppressed the expression and activity of MMP-2, which

is known to be involved in the process of angiogenesis. Moreover, previous studies have identified proliferin, a placental hormone, as a c-Jun-regulated angiogenic factor in fibrosarcoma cell lines (Toft et al., 2001). In breast cancer, activated c-Jun has been shown to be predominantly expressed at the invasive front and to be associated with angiogenesis (Vleugel et al., 2006).

2.1.3.6 Other putative c-Jun target genes in tumorigenesis

c-Jun has also been reported to regulate the transcription of phosphoinositide-dependent kinase 1 (PDK1), which is a kinase required for the activation of other kinases such as Akt and protein kinase C (PKC) (Lopez-Bergami et al., 2010). Akt and PKC signal transduction pathways, in turn, are important in tumor development and progression in many tumor types.

Dhar *et al.* (2004) identified by microarray analysis six AP-1-regulated genes that are critical in epithelial tumor promotion. These genes were TPA-inducible and suppressed by TAM67, three of them being transcription factors: c-Jun, high-mobility group A1 (HMGA1), and transcription factor IIb (TFIIb9), and the other three a translation initiation factor (eIF4a), an early regulator of the NF-kB signaling pathway (IkBa), and an expressed sequence tag (EST) with coding similarities to a mouse caveolar protein. Notably, HMGA1 binds to the AT-rich region in the minor groove of DNA, controlling gene transcription by changing DNA folding and recruiting other transcription-related factors (Watanabe et al., 2013; reviewed in Wang, Y. et al., 2019).

Notably, c-Jun has also been found to directly bind to MDM2 (mouse double minute 2 homolog) promoter to regulate MDM2 expression in colorectal cancer (Wang, B. et al., 2015). MDM2 has been identified to repress transcriptional activity of tumor suppressor p53. Wang *et al.* (2015) further showed that in colorectal cancer decreased miR-194 (microRNA-194) expression resulted in MAP4K4 (MAP kinase kinase kinase kinase 4) activation, which in turn led to activation of JNK and c-Jun. Similarly, also decreased miR200c has been reported to lead to activation of JNK2 and c-Jun in colorectal cancer, resulting in enhanced P-gp- (P-glycoprotein) mediated invasion and metastasis (Sui et al., 2014). In addition, TGF-β is known to promote p38α-dependent phosphorylation of c-Jun at Ser63, which leads to c-Jun activation and binding to the promoter of Snail1. Snail1 expression then induces migration and invasion in prostate cancer (Thakur et al., 2014). Gao *et al.* (2014), in turn, showed that c-Jun directly activates FUT1 (alpha 1, 2-fucosyltransferase 1) transcription by binding its promoter in ovarian cancer cells. FUT1 expression increases synthesis of Lewis Y glycan, which mediates

several tumor-promoting abilities, including cell proliferation, invasion, metastasis, and drug resistance (Gao, N. et al., 2014).

2.2 OTHER AP-1 PROTEINS

2.2.1 JunB and JunD

The Jun proteins share many structural and biochemical properties, but differ in their biological functions. All *jun* genes are intronless (www.ensembl.org). JunB, as well as c-Jun, contains a JNK-docking site, but JunB does not have a typical JNK phosphorylation site in its transactivation domain, and thus, JNK cannot activate it in the same manner as c-Jun (Jin, J. Y. et al., 2011).

The effect of JunB on neoplastic transformation is context-dependent such that JunB may function as a tumor suppressor or tumor promoter. JunB can have both cell division-inhibiting and cell division-promoting activities depending on the cell cycle stage and the environmental conditions (reviewed in Piechaczyk and Farras, 2008). JunB has been found to be downregulated in human high-grade prostate cancer and to have tumor suppressor activity in the context of *PTEN* loss (Thomsen et al., 2015). On the other hand, JunB may have an important role in promoting cell invasion, migration, and distant metastasis in head and neck squamous cell carcinoma, and cell invasion and angiogenesis in von Hippel-Lindau defective renal cell carcinoma (Hyakusoku et al., 2016; Kanno et al., 2012). JUNB has been shown to activate *Vegfa* (Schmidt et al., 2007), which is the master regulator of angiogenesis (Olsson et al., 2006).

JunB is often thought to be antagonistic to c-Jun. For example, dominant negative JunB mutant lacking the transactivating domain promotes epidermal malignancy, while the opposite is seen with the dominant negative mutant of c-Jun, TAM67 (Jin, J. Y. et al., 2011). Further, knocking down JunB by using a specific short hairpin RNA (shRNA) induces transcriptional expression of c-Jun in immortalized fibroblasts, leading to a significant increase in AP-1 activity (Gurzov et al., 2008). This may be due to negative regulation of c-Jun expression by JunB through competition for the AP-1 site within the *c-jun* promoter.

The regulatory mechanisms of JunD, by contrast, are different from the other AP-1 proteins. JunD is not regulated as an immediate early gene, but its mRNA is detectable in quiescent cells and the protein is degraded within 30 minutes following serum stimulation (reviewed in

Hernandez et al., 2008). In addition, JunD suppresses Ras-mediated transformation, whereas c-Jun cooperates with Ras to transform cells (Pfarr et al., 1994). In prostate cancer, JunD plays an important role in cell proliferation, and failure of JunD protein degradation may induce resistance to the proliferation inhibitory effects of TGF-β at advanced stages of cancer (Millena et al., 2016). Unlike c-Jun and JunB, JunD does not undergo ubiquitination-mediated proteasomal degradation or modification by the small ubiquitin-related modifier (SUMO) family proteins (reviewed in Piechaczyk and Farras, 2008; Hernandez et al., 2008).

2.2.2 Fos family

The Fos family of transcription factors includes c-Fos, FosB, Fra-1, Fra-2, and smaller FosB splice variants FosB2 and deltaFosB2 (reviewed in Milde-Langosch, 2005). Fos family members are not able to form homodimers, but they dimerize with Jun family members to form the AP-1 transcription factor complex. Like other AP-1 proteins, Fos family members have a bZIP for dimerization and DNA-binding, but only c-Fos and FosB proteins have a C-terminal transactivation domain. MAP kinases ERK1 and ERK2 phosphorylate c-Fos at Ser374 (reviewed in Roskoski, 2012). The functional domains and phosphorylation sites of c-Fos are shown in Figure 4. It was initially proposed that Fra-1, Fra-2, and FosB2 have an inhibitory function on AP-1 activity (reviewed in Tulchinsky, 2000). However, subsequent results have shown that although Fra1 and Fra2 lack the potent transactivation domains, they might be involved in the progression of many tumor types and have a positive effect on tumor invasion (Milde-Langosch et al., 2008; Milde-Langosch et al., 2004 reviewed in Milde-Langosch, 2005).

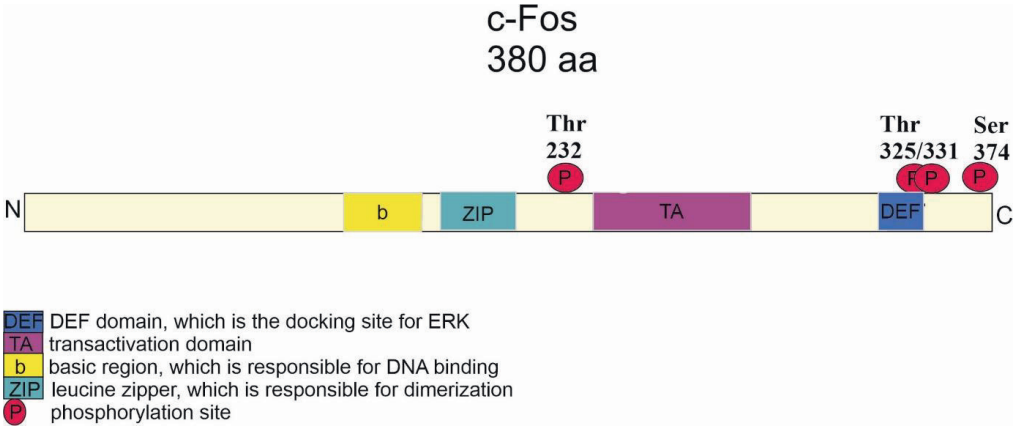


Figure 4. Structure of c-Fos (reviewed in Hess et al., 2004).

In addition to c-Jun, c-Fos is a main AP-1 protein in mammalian cells. It was first identified as the viral oncoprotein v-Fos in the Finkel-Biskis-Jinkins murine osteogenic sarcoma virus (FBJ-MSV) (Curran et al., 1983). Furthermore, c-Fos was found to be transforming in rat fibroblasts (Miller et al., 1984). Like c-Jun, c-Fos is an unstable protein that can be rapidly induced by mitogenic stimuli (Greenberg and Ziff, 1984). It is targeted for ubiquitination and degradation by lysine-specific demethylase 2B (KDM2B)-containing E3 ligase and can be stabilized by EGF-promoted phosphorylation, which dissociates c-Fos from its ubiquitin E3-ligase (Han et al., 2016). c-Fos overexpression in mice can cause osteosarcoma formation (Wang, Z. Q. et al., 1995). c-Fos is also frequently found to be overexpressed in human cancers such as cervical and thyroid cancers and oral squamous cell carcinoma (Cheung et al., 1997; Kataki et al., 2003; Dong et al., 2014).

2.2.3 ATF/CREB family

ATF1, ATF2, LRF1/ATF3, ATF4/CREB2, ATF5/ATFx, ATF6, B-ATF, JDP1, and JDP2 belong to the ATF/CREB family of bZIP transcription factors. They bind to the 8-base palindromic CRE promoters that respond to elevated cAMP. In addition, ATF2 binds to other elements, such as the AP-1 element, the proximal element of the IFN-γ promoter, the stress-response element of the ho-1 gene, and the UV-response element (reviewed in Vlahopoulos et al., 2008). The ATF/CREB family of transcription factors shares the ability to respond to environmental signals and maintains cellular homeostasis (Hai and Hartman, 2001). ATF2 regulates transcriptional responses associated with cell proliferation and differentiation, tumorigenesis, and apoptosis. JNK and p38 phosphorylate ATF2, as well as c-Jun, in response to cellular stress, which, in turn, enables the heterodimer formation. Indeed, c-Jun-ATF2 is a common dimer in oncogenic processes (reviewed in Vlahopoulos et al., 2008). ATF2 has been found to play a role in several cancers such as prostate and breast cancer, leukemia, neuroblastoma, and melanoma (reviewed in Vlahopoulos et al., 2008). In addition, many other ATF/CREB family members are also positively associated with cancer progression, e.g. ATF1 with human melanoma cells (reviewed in Leslie and Bar-Eli, 2005) and thyroid papillary carcinoma (Ghoneim et al., 2007), CREB with human lung cancer cell lines (Linnerth et al., 2005), and ATF6α with hepatocarcinogenesis (Arai et al., 2006).

JDP2 interacts with c-Jun, but also with the transcription factors ATF2 (Jin, C. et al., 2001) and CCAAT/enhancer-binding protein gamma (reviewed in Tsai et al., 2016). JDP2 can bind both TPA- and cAMP-response elements, resulting in the inhibition of transcription. JDP2 acts as a repressor at the AP-1 site, so its tumor suppressor action can be partially explained via a

decrease of c-Jun expression and an increase of JunB, JunD, and Fra2 expression (Heinrich et al., 2004). In addition, Heinrich et al. (2004) found JDP2 to be able to inhibit Ras-driven transformation of NIH 3T3 cells. JDP2 is widely expressed in normal mammalian tissues, and its activity is regulated by phosphorylation and SUMOylation (Wang, C. M. et al., 2017).

2.2.4 MAF family

The Maf transcription factors are members of the bZIP transcription factors belonging to the AP-1 superfamily. Mafs can be divided into two Maf families: so-called large Mafs, which include c-Maf, MafA, MafB, and NRL proteins (reviewed in Eychene et al., 2008), and small Mafs, including MafF, MafK, and MafG, which lack the N-terminal activation domain (reviewed in Katsuoka and Yamamoto, 2016; Kannan et al., 2012). Large Mafs are oncogenes and their transforming activity relies on overexpression (reviewed in Eychene et al., 2008). *c-maf* is the cellular homolog of *v-maf*, which is the transforming gene of the avian retrovirus AS42. *v-maf* was first isolated from spontaneous musculo-aponeurotic fibrosarcoma in chicken, and it can transform primary chicken embryo fibroblasts (CEFs) (Nishizawa et al., 1989). Overexpressed c-Maf has been found, for example, in half of the multiple myelomas, where it upregulates cyclin D2, CCR1 (leading to tumor survival and expansion), and integrin β7 (leading to increased tumor-stroma interactions). c-Maf can form homodimers or heterodimers with other bZIP transcription factors. c-Maf and MafA can form stable heterodimers with Jun and bind -TGC TGA C TCA GCA- DNA motif, which also contains an internal Jun:Fos site. Another palindromic DNA sequence where Maf preferentially binds is –TGC TGA CG TCA GCA-, and both of these sequences are Maf recognition elements (MAREs) (Kataoka et al., 1994).

3. POLYAMINES AND THEIR BIOSYNTHETIC ENZYMES

3.1 POLYAMINES AND THEIR FUNCTIONAL ROLES IN CELLS

Polyamines (putrescine, spermidine, and spermine) are small, organic polycations that have positive charges distributed along a conformationally flexible carbon chain (Figure 5). Thus, they interact with negatively charged macromolecules such as DNA, RNA, acidic proteins, and acidic phospholipids. Further, through their aliphatic hydrocarbon backbones polyamines are able to establish hydrophobic interactions as well. Polyamines have thus the capability to interact simultaneously with different macromolecular structures at the same time (reviewed in Sanchez-Jimenez et al., 2019). By binding to cellular macromolecules, polyamines have an important role in the maintenance of chromatin structure, membrane stability, and intracellular

ionic balance (reviewed in Igarashi and Kashiwagi, 2019). In addition, they regulate many essential cellular functions such as gene expression, cell proliferation, and apoptosis. Further, polyamines play an important role in signaling processes, including the regulation of both the expression and activation state of MAP kinases (reviewed in Ramani et al., 2014).

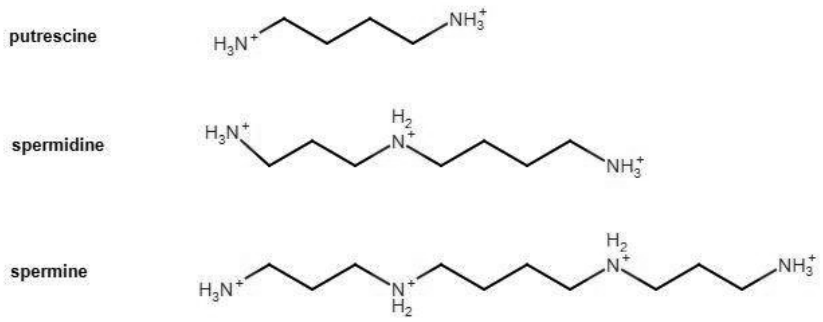


Figure 5. Structure of polyamines.

Eukaryotic cells need polyamines for normal cell proliferation and development (reviewed in Tabor and Tabor, 1984; Cohen, 1998). In normal physiological conditions, intracellular polyamine concentration is tightly controlled and dysregulation of the polyamine biosynthetic pathway leads to different pathological conditions, including neurodegenerative diseases, digestive diseases, and cancer (reviewed in Park, M. H. and Igarashi, 2013; Nowotarski et al., 2013; Sanchez-Jimenez et al., 2019). Indeed, elevated levels of polyamines have been associated with several human cancers such as breast, colon, and skin cancers (Manni et al., 1995; Gilmour, 2007; Upp et al., 1988). Most of the hallmarks of cancer described by Hanahan and Weinberg (2011) are affected by polyamines, e.g. they improve the ability of cancer cells to invade and metastasize, but also decrease the antitumor immune functions of immune cells (reviewed in Sanchez-Jimenez et al., 2019).

3.2 BIOSYNTHETIC ENZYMES OF POLYAMINES

The two main regulatory enzymes of polyamine biosynthesis are ODC and AdoMetDC (reviewed in Miller-Fleming et al., 2015). Figure 6 shows a simplified diagram of the locations of ODC and AdoMetDC enzymes in the biosynthetic pathway of polyamines.

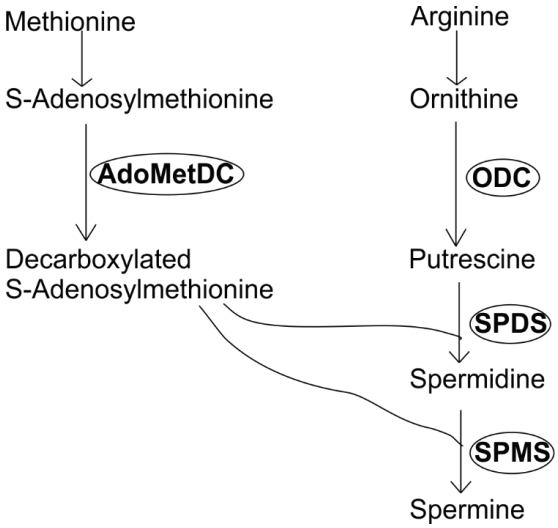


Figure 6. Simplified diagram of polyamine biosynthesis. The enzymes are indicated in boldface in ovals. AdoMetDC: S-adenosylmethionine decarboxylase; ODC: ornithine decarboxylase; SPDS: spermidine synthase; SPMS: spermine synthase.

3.2.1 Ornithine decarboxylase (ODC)

The first and rate-limiting step in polyamine biosynthesis is catalyzed by ODC, decarboxylating L-ornithine into putrescine, the first polyamine. ODC is a ubiquitously expressed enzyme, which is found in all types of cells. It is controlled in many ways, including transcriptional and translational regulation and protein turnover. The transcriptional control occurs in response of the *Odc* gene promoter region to hormones, growth factors, tumor promoters, and oncoproteins such as MYC and RAS (Hölttä et al., 1988; reviewed in Pegg, 2006). Indeed, *Odc* is a well-known target of the c-MYC oncoprotein, which increases the activity of the MYC/MAX transcription complex that binds to the *Odc* promoter (Pena et al., 1993; Pena et al., 1995; Tobias et al., 1995; Bello-Fernandez et al., 1993). In quiescent cells, the MYC antagonist MNT/MAX complex occupies this site (Nilsson and Cleveland, 2004). At the translational level, one interesting example of the negative translational control of ODC is that the translation of ODC mRNA is reduced by increased polyamine level (reviewed in Pegg, 2006).

The degradation rate of ODC is also tightly regulated, and the half-life of ODC is very short (Russell and Snyder, 1969). In response to intracellular polyamine levels, ODC degradation is controlled by an autoregulatory loop involving specific polypeptides acting as ODC inhibitors called antizymes (Az) (reviewed in Hayashi et al., 1996; Kahana, 2009; Coffino, 2001) and their counteractors called antizyme inhibitors (AzI) (reviewed in Kahana, 2009; Kahana, 2018; Ramos-Molina et al., 2018). In mammalian cells, there are three well-characterized homologous antizymes Az1, Az2, and Az3, and one putative member termed Az4, and two antizyme inhibitors AzI1 and AzI2. In its active state, ODC is a loosely bound homodimer that easily dissociates into monomers. Az binds non-covalently to ODC subunits with higher affinity than the ODC subunits to each other, inactivating ODC and targeting it to ubiquitin-independent degradation by proteasome 26S (reviewed in Pegg, 2006; Ramani et al., 2014; Kahana, 2018). AzI is highly homologous to ODC, but lacks ornithine-decarboxylating activity. It can bind with higher affinity to Az than Az binds to ODC, rescuing ODC from the interaction with Az and degradation (reviewed in Kahana, 2018).

Previously, ODC has been shown to induce transformation of immortalized rodent fibroblasts, and further induce tumors in *nude* mice (Auvinen et al., 1992; Auvinen et al., 1997). Because ODC and polyamine levels have been found to be highly elevated in proliferating tumor cells, inhibition of ODC is speculated to be a good way to combat cancer (reviewed in Shantz and Levin, 2007).

3.2.2 S-adenosylmethionine decarboxylase (AdoMetDC)

AdoMetDC catalyzes the decarboxylation of S-adenosylmethionine, which acts as an aminopropyl donor in the biosynthesis of spermidine and spermine. AdoMetDC is another key enzyme in the biosynthesis of polyamines, regulating the formation of the higher polyamines spermidine and spermine. It is also highly regulated, in the same way as ODC (Suzuki et al., 1993; Shantz et al., 1992; reviewed in Pegg et al., 1998; Pegg, 2009). The cellular need for polyamines determines the amount and activation of the AdoMetDC protein. Interestingly, increased putrescine content increases the activation of AdoMetDC, while increased spermidine and spermine content decreases the transcription of AdoMetDC gene (probably through a polyamine-responsive element) and the translation of AdoMetDC mRNA [through ORFs (open reading frames) in the 5'-UTR (untranslated region) of the mRNA] (reviewed in Pegg, 2009). Similarly to ODC, AdoMetDC has a fast turnover rate via degradation by the 26S proteasome (reviewed in Ramani et al., 2014; Pegg, 2009).

AIMS OF THE STUDY

The aim of this thesis was to examine the role of transcription factor c-Jun in transformation of different cell types. c-Jun is constitutively activated in many transformed cell lines and human cancers, including ODC-transformed mouse fibroblasts and human melanoma cells arising from various genetic alterations.

Specific aims were as follows:

- To examine the potential transforming activity of AdoMetDC, the other key regulatory enzyme of polyamine biosynthesis, in addition to ODC.
- To evaluate the significance of c-Jun phosphorylation and activation for ODC-, AdoMetDC-, and Ha-*ras*-induced cell transformation of mouse fibroblasts.
- To identify potential transformation-relevant genes regulated by c-Jun in ODC-, AdoMetDC-, and RAS-transformed mouse fibroblasts and human melanoma cells, and to assess their functional roles in these cells.

MATERIALS AND METHODS

4. CELL CULTURE (I-IV)

Mouse fibroblast cell lines used in the study are presented in Table 2 and human cells in Table 3. All cells were cultured at 37ºC in a 5% CO_2 atmosphere.

Table 2. Mouse cell lines.

Name	Parental cell line	Vector and insert	Publication
NIH3T3	-	-	II
4N	NIH3T3	pLTRpoly	I, III
Amdc	NIH3T3	pLTRpoly and AdoMetDC sense	I, III
Amdc-as*	NIH3T3	pLTRpoly and AdoMetDC antisense	I
N1	NIH3T3	pSV2neo	II, IV
Odc	NIH3T3	pLTRpoly and human ODC cDNA	II, IV
Odc-n**	Odc	-	II, IV
E4	NIH3T3	pGEJ6.6 (c-Ha-*ras*^{val12})	II, IV
NIH3T3-pLRT-TAM67§	NIH3T3	pLRT-TAM67	II
Amdc-pLRT-TAM67§	Amdc	pLRT-TAM67	III
Amdc-as-pLRT-TAM67§	Amdc-as	pLRT-TAM67	-
Odc-pLRT-TAM67§	Odc	pLRT-TAM67	II, IV
Odc-n-pLRT-TAM67	Odc-n	pLRT-TAM67	II
E4-pLRT-TAM67	E4	pLRT-TAM67	II
Amdc-pLRT-LOX§	Amdc	pLRT-human LOX	-
Odc-pLRT-LOX§	Odc	pLRT-human LOX	IV

*Spermidine was used at a low concentration (1 μM) in Amdc-as cell cultures to prevent the counter-selection of cells expressing adequate levels of AdoMetDC antisense-mRNA to block the synthesis of essential polyamines.
**Odc-n cells are derived from the Odc cell line-induced tumors of *nude* mice.
§Doxycycline (1 μg/ml) was used in the growth medium to induce TAM67 or LOX expression.

The NIH3T3 cell line was from the American Type Culture Collection. Normal mouse fibroblast cell lines (NIH3T3, 4N, and N1) were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin, and 5% newborn calf serum (NBCS; Life Technologies, Inc.). Transformed mouse fibroblast cell lines (Amdc, Amdc-as, Odc, Odc-n and E4) were cultured as the normal fibroblast cell lines, except that 5% fetal bovine serum (FBS; Bioclear) was used instead of NBCS.

Transformed fibroblast cell lines were stably transfected with a tetracycline-inducible expression system of the transactivation domain deletion mutant of c-Jun (pLRT-TAM67) or human lysyl oxidase (pLRT-LOX), and normal fibroblast NIH3T3 cells were transfected with

pLRT-TAM67. These cell lines (Amdc-/ Amdc-as-/ Odc-/ Odc-n-/ E4-pLRT-TAM67/LOX) were cultured in αMEM (Invitrogen), supplemented with 2 mM L-glutamine, 50 μg/ml gentamicin, and Tet system-approved fetal bovine serum (Tet-FBS; Clontech). NIH3T3-pLRT-TAM67 cells were cultured with NBCS.

Table 3. Human cells.

Name	Description	Publication
HT-1080	Fibrosarcoma cell line	III
42V	Primary melanocytes	IV
Mela3	Primary melanocytes	IV
Mela TN45	Primary melanocytes	IV
EL29	Primary melanoma cells	IV
WM115	Primary melanoma cell line (vertical growth phase)	IV
WM793	Primary melanoma cell line (vertical growth phase)	IV
WM239	Metastatic melanoma cell line (lymph node metastasis)	IV
MM170	Metastatic melanoma cell line (lymph node metastasis)	IV
SK-MEL-28	Metastatic melanoma cell line (skin metastasis)	IV
BLM	Metastatic melanoma cell line (lung metastasis)	IV
SK-MEL-103	Metastatic melanoma cell line	IV
SK-MEL-147	Metastatic melanoma cell line	IV
HMVEC	Microvascular endothelial cells	IV
HES	Primary embryonic skin fibroblasts	IV
Fibroblast	Primary foreskin fibroblasts	IV
Fibroblast	Primary adult skin fibroblasts	IV

For a detailed description of human cells, culture conditions, and references see Study IV (for HT-1080 see Study III). Primary human melanocytes 42V, Mela3, and Mela TN45 were grown as previously described (Alanko et al., 1999; Kääriäinen et al., 2006; Soikkeli et al., 2010). Human fibrosarcoma cell line HT-1080 and metastatic melanoma cell lines SK-MEL-103, SK-MEL-147, and BLM were grown in DMEM containing 10% FBS and antibiotics. Human primary embryonic skin fibroblasts (HES), melanoma cells isolated from a primary tumor (EL29), melanoma cells isolated from a primary melanoma of vertical growth phase (WM115 and WM793), and cells from a metastatic melanoma (WM239, MM170, and SKMel-28) were cultured in RPMI 1640 containing 10% FBS and antibiotics. Human microvascular endothelial cells (HMVECs) were grown in growth factor-supplemented medium 131 (Gibco/Invitrogen).

5. PATIENT SAMPLES (IV)

Benign nevi and primary cutaneous melanomas were obtained by surgical excision at Helsinki University Central Hospital. Informed consent was given by the patients. The protocols for

taking the specimens were approved by the Ethics Committees of Helsinki University Central Hospital.

6. RNA ANALYSES

6.1 MICROARRAY ANALYSIS (III, IV)

Polyadenylated RNAs were extracted from the exponentially growing Amdc-pLRT-TAM67, Amdc-as-pLRT-TAM67, and Odc-pLRT-TAM67 cells by oligo(dT) cellulose affinity chromatography (Roche Diagnostics). TAM67 expression was induced (+dox) or not (-dox) 3 and 6 days before RNA extraction by 1 μg/ml doxycycline. Polyadenylated RNAs were reverse-transcribed with Cy3 and Cy5 end-labeled random 9-mers to generate fluorescent single-stranded cDNA probes. These were then competitively hybridized to and analyzed with Mouse GEM2/Unigene1 cDNA LifeArrays (Incyte Genomics) containing 9596 expressed elements representing 9307 unique genes or clusters.

Total RNAs were extracted with RNeasy Kit (Qiagen) from Amdc-pLRT-TAM67 and Odc-pLRT-TAM67 cells induced (+dox) or not (-dox) with 1 μg/ml doxycycline for 3 or 5 days, and from N1, Odc, and E4 cells at 2-3 days after plating in the serum containing media. Biotinylated cRNA targets were produced from the RNAs and hybridized to Affymetrix oligonucleotide MOE430 set arrays containing more than 30,000 gene probes.

6.2 NORTHERN BLOT ANALYSIS (I, IV)

The AdoMetDC mRNA levels of the normal 4N fibroblast, Amdc, and Amdc-as cells, and the LOX mRNA levels of the Odc-pLRT-LOX clones 2 and 6 were confirmed by Northern blot analysis. LOX expression was induced (+dox) or not (-dox) with 1 μg/ml doxycycline for 3 days in the Odc-pLRT-LOX clones. Polyadenylated RNA samples were resolved in agarose gels and transferred to Hybond-N nylon membrane (Amersham Pharmacia Biotech). [^{32}P]dCTP-labeled AdoMetDC and LOX antisense cDNA probes were hybridized to membranes. [^{32}P]dCTP-labeled β-actin cDNA was used as a control and hybridized to check the β-actin expression levels. Hybridization signals were detected and quantified by using Fuji's imaging plates and MacBAS 2.5 software.

6.3 REVERSE TRANSCRIPTION-PCR (RT-PCR) ANALYSIS (III, IV)

The expression levels of the identified, c-Jun regulatable, candidate genes (*Itgα6*, *Itgβ7*, and *Lox*) in the transformed fibroblast cell lines and the genes related to these molecules (*Itgβ1*, *Itgβ4*, *Loxl1*, *Loxl2*, *Loxl3,* and *Loxl4*) were confirmed by reverse transcription (RT)-PCR analysis. In addition, the expression levels of *LOX*, *LOXL1*, *LOXL2*, *LOXL3,* and *LOXL4* in melanoma cells were analyzed by RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as a control with mouse cells and β-actin (ACTB) with human cells. The mouse- and human-specific primers and PCR conditions used are shown in Tables 4 and 5, respectively.

Table 4. Mouse-specific PCR primer sequences and PCR conditions used in Studies III and IV.

Gene	Orientation	Sequence 5' to 3'	Annealing (°C)	Cycles*
Gapdh	F	GGGTGTGAACCACGAGAAAT	59	20
	R	GGTCCTCAGTGTAGCCCAAG		
Cathepsin L	F	ATGAGGAATTCAGGCAGGTGGTGA	56	18
	R	ACTCAGTGAGATCAGTTTGCCGGT		
Itgα6	F	TGGAGGTACAGTTGTTGGTGAGCA	55	20
	R	AAACACCGTCACTCGAACCTGAGT		
Itgβ1	F	TCGCTGATTGGCTGGAGGAATGTA	57	20
	R	TCCTGCAGTAAGCGTCCATGTCTT		
Itgβ4	F	AGGGAGGCTGGCTTTCAATGTAGT	55	25
	R	TTCACCAGGTGCTCAGTGTCATCA		
Itgβ7	F	AGTGTGCGACTGTAACTGTGGTGA	53	23
	R	ACTCTGCACAATCCCTGTACTGCT		
Lox	F	TGGCACAGCTGTCACCAACATT	61	25
	R	ACAGAAGCTTGCTTTGTGGCCT		
Loxl1	F	GCATCCACATACGTGCAGAG	62	25
	R	GGTCGTAGTGGCTGAACTCG		
Loxl2	F	GTGCCAACTTTGGAGAACAAGGCA	61	30
	R	TTGTACATCCAGATGCGGTAGCCA		
Loxl3	F	AGAACATCACAGCTGAGGAC	64	29
	R	GCTCATCACCACCTCAGTTAC		
Loxl4	F	AAGTGGTGATGAGTGGAGTTCGCT	62	30
	R	TTTCCTCGTGAGCACAGTACAGCA		

*The number of cycles was optimized to be in the linear range.

F=forward, R=reverse

Table 5. Human-specific PCR primer sequences and PCR conditions used in Study IV.

Gene	Orientation	Sequence 5' to 3'	Annealing (°C)	Cycles*
ACTB	F	GCTCGTCGTCGACAACGGCTC	55	25
	R	CAAACATGATCTGGGTCATCTTCTC		
LOX	F	ATGATCACAGGGTGCTGCTCAGAT	61	20
	R	GTGTGCAGTACATGCAAATCGCCT		
LOXL1	F	ATCCACTTATGTGCAGAGAGCCCA	62	24
	R	AGTCGATGTCCGCATTGTAGGTGT		
LOXL2	F	AGCTTCTGCTTGGAGGACACAGAA	60	22
	R	GCGGCTCCTGCATTTCATGATGTT		
LOXL3	F	TGTTGTACTGTGCTGCGGAAGAGA	60	22
	R	GAAGGCATCACCAATGTGGCAGTT		
LOXL4	F	GCATGACATTGATTGCCAGTGGGT	64	20
	R	ATGAGGTTGTTCCTGAGACGCTGT		

*The number of cycles was optimized to be in the linear range. F=forward, R=reverse.

7. PROTEIN ANALYSES

7.1 WESTERN BLOTTING (I-IV)

Whole-cell lysates, secreted proteins from concentrated serum-free conditioned media, and immunoprecipitated proteins in 1x Laemmli sample buffer were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were probed with primary antibodies (Table 6) and horseradish peroxidase conjugated secondary antibodies. Blots from human fibroblasts and melanoma cell lines were quantified using Image Studio Lite.

Table 6. Antibodies used in Studies I-IV.

Target	Clone/Code	Source	Application*	Manufacturer**	Publication
Actin (Ab-1)	JLA20	mouse	WB	Merck	II, III
c-Jun/AP-1	Ab-1	rabbit	WB	Merck	I, II, III
c-Jun	L70B11	mouse	WB	CST	III
	ab31419	rabbit	WB	Abcam	III
	E254	rabbit	IHC	Epitomics	III
p-c-Jun	9164	rabbit	IHC	CST	III
p-c-Jun (Ser63)		rabbit	WB	NEB	I
Erk1/Erk2		mouse	WB	Zymed	I
Flag-Tag	M2	mouse	WB	Sigma-Aldrich	II
Ha-Tag	12CA5	mouse	WB	BM	II
Itgα6	MA6	rat	IF, IP	Chemicon	III
	GoH3	rat	FC, IP, FB	RD systems	III
	H-87	rabbit	IHC	Santa Cruz	III
	3750	rabbit	WB	CST	III
	HPA012696	rabbit	WB	Atlas Antibodies	III
Itgβ1	AB1952	rabbit	WB	Chemicon	III
	HA2/5	hamster	FB	Fitzgerald	III
	6S6	mouse	FB	Chemicon	III
Itgβ4	346-11A	rat	WB	BD Pharmingen	III
	M126	mouse	WB	Abcam	III
Itgβ7	FIB27	rat	FB	BD Pharmingen	III
	M293	rat	IF	BD Pharmingen	III
JNK1 (FL)	F-3	mouse	WB	Santa Cruz	I
JNK1/JNK3	C-17	rabbit	IP	Santa Cruz	I
Lysyl oxidase	L4669	rabbit	WB	Sigma-Aldrich	IV
Lysyl oxidase-like 2	ab96233	rabbit	WB	Abcam	IV
Lysyl oxidase propeptide	NB110-41568	rabbit	WB	Novus Biologicals	IV
MKK4/SEK1/JNKK1	M7433	rabbit	WB	Sigma-Aldrich	I, II
p38	C-20	rabbit	WB	Santa Cruz	II
P-p38 (Thr180/Tyr182)		rabbit	WB	Cell signalling Technology	II
α-tubulin	600-401-880	rabbit	WB	Rockland	III

*WB=Western blot, IHC=immunohistochemistry, IF=immunofluorescent stain, IP=immunoprecipitation, FC=flow cytometry, FB=function blocking, **CJT=Cell Signaling Technology, NEB=New England Biolabs, Inc, BM=Boehringer Mannheim.

7.2 IMMUNOFLUORESCENT STAINING (III)

Amdc cells were grown on glass coverslips, which were then fixed with 3.5% paraformaldehyde and permeabilized with 0.1% Triton X-100. In the stainings, anti-integrin α6 or β7 primary antibodies and FITC-conjugated goat anti-rat secondary antibody were used.

7.3 IMMUNOPRECIPITATION ANALYSIS (III)

To determine the binding partners of integrin α6, Amdc cells were lysed and 1.5 mg proteins were incubated with integrin α6 antibody (MA6 or GoH3) overnight at 4°C. Immunocomplexes were collected using anti-rat IgG agarose (Sigma), washed, and eluted. The candidate binding partners integrin β1 and β4 were then analyzed by Western blotting.

7.4 IMMUNOHISTOCHEMISTRY (III)

Paraffin-embedded human tissue samples of low- and high-grade fibrosarcomas, and normal esophagus and bronchus as a control, were used in immunohistochemical staining of integrin α6. In addition, c-Jun and phospho-c-Jun (Ser73) were analyzed by immunohistochemical analyses in different grade human sarcomas.

7.5 FLOW CYTOMETRY (III)

The flow cytometer (FACScan; Becton Dickinson) was used to analyze the cell surface levels of integrin α6 in 4N and Amdc cells. The cell suspensions were first incubated with anti-integrin α6 antibody or isotype control antibody, washed, and incubated with secondary biotinylated goat anti-rat antibody, and finally with r-phycoerythrin-streptavidin (Vector Laboratories, Inc.).

8. FUNCTIONAL ANALYSES

8.1 TRANSFECTION EXPERIMENTS (I-IV)

8.1.1 Plasmids (I, II)

Plasmids used in the study are summarized in Table 7. To investigate the effect of overexpression of AdoMetDC, pLTRpoly vector with human AdoMetDC cDNA in sense or antisense orientations together with *neo* selection marker were transfected into normal NIH 3T3 mouse fibroblast cells and Rat1 fibroblasts using LipofectAMINE. Stable cell lines were generated and the names of the cell lines used in further studies are as follows: 4N (transfected

with empty pLTRpoly vector and pSV2*neo*), Amdc (AdoMetDC-pLTRpoly and pSV2*neo*), and Amdc-as (AdoMetDC-as-pLTRpoly and pSV2*neo*).

Table 7. Plasmids.

Plasmid	Description	Manufacturer/ Provider/ Reference	Publication
pLTRpoly	empty vector	American Type Culture Collection	I
AdoMetDC-pLTRpoly	S-adenosylmethionine decarboxylase		I
AdoMetDC-as-pLTRpoly	S-adenosylmethionine decarboxylase in antisense orientation		I
pSV2*neo*	neomycin selection marker		I
pcDNA3	empty vector	Invitrogen	I, II
DN JNK1	FLAG-JNK1 (APF), dominant negative JNK1	Roger J. Davis, James R. Woodgett (Derijard et al., 1994)	I, II
DN SEK1	SEK1 (AL), dominant negative SEK1/MKK4	Roger J. Davis, James R. Woodgett (Yan et al., 1994)	I, II
DN MKK4	Flag-MKK4 (Ala), dominant negative MKK4	(Whitmarsh et al., 1997)	II
pMT 108	c-Jun	(Treier et al., 1995; Papavassiliou et al., 1995)	II
pMT 111	c-Jun^{S63,73A}	(Treier et al., 1995; Papavassiliou et al., 1995)	II
pMT 161	c-Jun^{S63,73A,T91,93A}	(Treier et al., 1995; Papavassiliou et al., 1995)	II
pCMV	empty vector	(Brown et al., 1993)	I
pCMV-TAM67	deletion mutant of c-Jun	(Brown et al., 1993)	I, II
pBabe puro	puromycin selection marker	(Morgenstern and Land, 1990)	I,II
pLRT-TAM67	inducible deletion mutant of c-Jun		II, III, IV
pLRT-LOX	inducible lysyl oxidase		IV
pLRT	empty inducible vector	(Watsuji et al., 1997)	IV

To examine the significance of JNK activation in the transformed Amdc, Odc, and E4 cells, empty pcDNA3 vector or dominant negative mutants of JNK1 or SEK1/MKK4 were transfected into the cells together with a pBabe puro selection marker, using LipofectAMINE Plus (Life Technologies). The cells were selected for resistance to puromycin, and pools of transfectants as well as individual clones were isolated.

To examine the role of c-Jun phosphorylation and activation in Odc and E4 cells, plasmids with normal c-Jun (pMT 108) as a control, phosphorylation site mutants of c-Jun (pMT 111 and pMT 161), and transactivation domain deletion mutant of c-Jun (pCMV-TAM67) were transfected together with a pBabe puro selection marker. In addition, empty pCMV vector and pCMV TAM67 together with a pBabe puro were transfected into Amdc and Amdc-as cells.

8.1.2 Tetracycline-inducible expression system (II-IV)

Tetracycline-inducible expression system of TAM67 and LOX were generated by inserting these cDNAs into reverse tetracycline-regulated retroviral vector pLRT (Watsuji et al., 1997). The pLRT-TAM67 plasmid was transfected into the normal NIH 3T3 mouse fibroblasts, Odc, Odc-n, E4, and Amdc cells, and the pLRT-LOX plasmid into the Odc and Odc-n cells using LipofectAMINE plus or LipofectAMINE 2000 (Invitrogen). Transfected cells were selected by Blasticidin (Invitrogen) and several clones were picked up. The cloned cells were tested for their inducibility of TAM67 or LOX expressions by 1 μg/ml doxycycline (Sigma). The best clones for further investigation were selected by morphological analyses and immunoblotting (TAM67 expression), and Northern blotting, RT-PCR, and immunoblotting (LOX expression).

8.1.3 siRNA and shRNA (III, IV)

To knock down c-Jun expression in Amdc cells, small interfering RNA (siRNA) oligonucleotide pool to c-Jun (Santa Cruz Biotechnology) and c-Jun short hairpin RNA (shRNA) construct (kindly provided by Dr. Kazushi Inoue, Wake Forest University Health Sciences, Winston-Salem, NC) were used. siRNA-A was used as a control for the former and an empty pSuper.retro.puro vector for the latter. Transfections were performed using OligofectAMINE (siRNAs), LipofectAMINE 2000 (Invitrogen), and Fugene 6 (shRNAs) (Roche Diagnostics) reagents.

To knock down the expression of LOX or LOXL2 in WM793 and SK-MEL-147 melanoma cells, MISSION® shRNA Lentiviral particles were used (Sigma-Aldrich). The clone TRCN0000045991 (SHCLNV-NM_002317) targeting LOX, clones TRCN0000046195 and TRCN0000046197 targeting LOXL2 (SHCLNV-NM_002318), and MISSION® Non-Mammalian shRNA Control Transduction Particles (SHC002V) were used as a control.

8.2 ACTIVITY ASSAYS (I-III)

8.2.1 AdoMetDC and ODC assays (I, II)

AdoMetDC and ODC activities were assayed by measuring the release of $^{14}CO_2$ from S-adenosyl-L-(carboxyl ^{14}C) methionine or L-(1-$^{14}CO_2$) ornithine, respectively, as previously described (Hölttä et al., 1988).

8.2.2 MAPK assay (I)

The activity of MAPK was assayed after its immunoprecipitation from 1 mg of soluble proteins with a polyclonal MAPK R2 antibody, and using ATP, (γ-^{32}P)ATP, and MAPK substrate peptide (APRTPGGRR) (Paasinen-Sohns and Hölttä, 1997).

8.2.3 JNK assay (I)

JNK activity was studied by using anti-JNK1 antibody and c-Jun(1-169)-GST fusion protein as a substrate (Paasinen-Sohns and Hölttä, 1997). On the solid phase kinase assay (Hibi et al., 1993), the JNKs were purified by their binding to agarose-conjugated substrate GST-c-Jun. For the kinase reaction, ATP and (γ-^{32}P)ATP were added and the phosphorylation of GST-c-Jun was analyzed by gel electrophoresis and quantification by autoradiography.

8.2.4 Cathepsin L assay (III)

The activity of cathepsin L was assayed as previously reported (Ravanko et al., 2004), in the presence of heparin. The possible interrelationship between integrin α6 and cathepsin L was evaluated by utilizing a neutralizing integrin α6 antibody. The effect of the antibody on cathepsin L activity was tested by incubating it with Amdc cells prior to and during culture both on plastic plates and on BD Biocoat Thin Layer Matrigel dishes. The growth medium was filtered to get rid of the antibody, and the secreted proteins were then concentrated and assayed for cathepsin L activity.

8.2.5 Adhesion assay (III)

The ability of Amdc cells to adhere on laminin- or fibronectin-coated plates was analyzed in the presence or absence of neutralizing antibodies to integrin subunits α6, β1, and β7 and isotype control antibody. The cells were pre-incubated (or not) with the neutralizing antibodies for 30 min and then allowed to adhere on laminin or fibronectin surfaces for another 30 min at 37°C. The cells were then washed, trypsinized, and counted with Coulter particle counter (Beckman Coulter).

8.2.6 Analysis of cell growth (II-IV)

The effects of TAM67 or LOX expression on the growth of Odc-pLRT-TAM67, E4-pLRT-TAM67, and NIH3T3-pLRT-TAM67, or Odc-pLRT-LOX cells, respectively, were evaluated. The cells were grown in the absence or presence of 1 μg/ml doxycycline, and counted at 24-h intervals over 4 days (TAM67) or after 3 and 5 days (LOX) with a Coulter particle counter (Beckman Coulter). To determine the significance of integrin subunits α6, β1, and β7 in the proliferation of Amdc cells, function-blocking antibodies of these integrins or isotype control antibody (Table 6) were incubated with the cells 30 min before plating and during growth. The cells were then counted after 3 and 6 days with a Coulter particle counter.

8.2.7 JNK inhibitors in cell culture (II)

The effects of two different cell-permeable JNK inhibitors, L-stereoisomer of JNK peptide inhibitor 1 (L-JNK inhibitor 1) and JNK inhibitor II (Table 8), were investigated in Odc cells. Peptide inhibitor and its control (L-TAT) were added daily to the cultures. DMSO (0.1%) was used as a control for JNK inhibitor II.

Table 8. Inhibitors and control used in Studies I-IV.

Inhibitor/ control	Target	Concentration	Manufacturer	Publication
L-stereoisomer of JNK peptide inhibitor 1 (L-JNK inhibitor 1)	JNK1, JNK2	1-25 μM	Alexis Biochemicals	II
L-stereoisomer of TAT control peptide (L-TAT)	-	1-25 μM	Alexis Biochemicals	II
JNK inhibitor II (SP600125)	JNK1, JNK2, JNK3	0.1-10 μM	Calbiochem	II
LOX inhibitor B-aminopropionitrile (BAPN)	LOX family	250/500 μM	Sigma-Aldrich	IV

8.2.8 Soft agar growth assay (I, II)

The ability of different cells, 4N as control, Amdc, Amdc-as (with 1 μM spermidine), Amdc cells transfected with pCMV-TAM67, and Odc-pLRT-TAM67 cells with inducible TAM67, to grow anchorage independently in the soft agar was evaluated. TAM67 expression was induced or not with 1 μg/ml doxycycline. For the assay, the cells in the growth medium with serum were mixed with agar (Difco) to yield a 0.35% agar mixture, which was then overlaid on 0.7% bottom agar and covered with growth medium. The colony formation was followed for 2-4 weeks, replenishing the growth medium twice a week.

8.2.9 Matrigel invasion assay (II-IV)

The ability of Odc-pLRT-TAM67, E4-pLRT-TAM67, Amdc-pLRT-TAM67, and Odc-pLRT-LOX cells to invade when TAM67 or LOX expression was induced or not with 1 μg/ml

doxycycline was examined by using 3D growth factor-reduced Matrigel (BD Biosciences). Further, the effect of lysyl oxidase inhibitor B-aminopropionitrile (BAPN) (see Table 8) on invasion of melanoma cell lines WM793 and SK-MEL-147 cultured with or without human primary embryonic skin fibroblasts (HES) was evaluated. For these co-cultures, the HES and melanoma cells WM793 and SK-MEL-147 were labeled with Celltracker Green CMFDA or Celltracker Red CMTPX (Life Technologies), respectively. In addition, the effect of BAPN to Odc-pLRT-LOX cells grown in Matrigel with or without doxycycline was examined. In these assays, doxycycline and BAPN were added to both the matrix and medium. Cells were allowed to grow in Matrigel for 1-5 days before photographing.

To also analyze the effect of Itgα6, β1, or β7 on cell invasion, neutralizing antibodies to these integrins or isotype control antibodies were preincubated with Amdc cells and human HT-1080 fibrosarcoma cells for 30 min before the invasion assays in Matrigel. The antibodies were also added to both the matrix and medium.

8.2.10 Tumorigenicity assay (I, II)

In the tumorigenicity assay, 4N (control), Amdc, Amdc-as, and Amdc-as cells with spermidine or Odc-pLRT-TAM67 cells were inoculated subcutaneously into both flanks of *nude* mice. The tumor formation of 4N, Amdc, Amdc-as, and Amdc-as with spermidine was followed by caliper measurements two to three times a week until reaching ethical limits (with Amdc and Amdc-as cells reached in 10 days). The tumor growth of the normal 4N cells was followed for up to 62 days. The mice inoculated with Odc-pLRT-TAM67 cells received (or not) 1 μM doxycycline in their drinking solution for TAM67 induction. These mice were sacrificed after 17 days and the tumors were weighed.

RESULTS AND DISCUSSION

9. C-JUN IS RELEVANT FOR CELL TRANSFORMATION INDUCED BY ADOMETDC (I), ODC, AND HA-*RAS* (II)

9.1 ADOMETDC-, ODC-, AND HA-*RAS*-TRANSFORMED CELLS

In this study, we investigated whether, in addition to ODC, also the other key regulatory enzyme of polyamine biosynthesis, AdoMetDC, is involved in cell transformation. To this end, we transfected AdoMetDC cDNA in sense and antisense orientations into NIH3T3 fibroblasts and Rat1 cells. Overexpression of AdoMetDC was also found to transform these rodent fibroblasts and induce highly invasive tumors in *nude* mice. AdoMetDc activity was 30- to 40-fold higher in AdoMetDC-transfomed cells than in parental cells. Surprisingly, in addition to AdoMetDC, also the expression of AdoMetDC cDNA in the antisense orientation and with 1 μM spermidine in the culture medium led to cell transformation. Notably, these latter cells had similar polyamine biosynthetic enzyme and polyamine patterns to those of the ODC-transformed cells, with an enhanced ODC activity and an elevated putrescine content. The increase in ODC activity in the AdoMetDC antisense cells is probably a compensatory mechanism. These transformed cells were also able to induce highly invasive tumors in *nude* mice.

In subsequent studies of the molecular mechanisms of transformation, we used NIH3T3 mouse fibroblast cell lines transformed by AdoMetDC, ODC, and c-Ha-*ras*^{Val12} (Amdc, Odc, and E4 cells, respectively). In our previous studies, we had obtained evidence that the proto-oncogene c-Jun is constitutively active in Odc cells (Hölttä et al., 1998). Furthermore, c-Jun had been shown to become transcriptionally activated by the phosphorylation of Ser63 and Ser73 in Ras-transformed cells and to be required for transformation (Johnson, R. et al., 1996). Ras proto-oncogenes (K-, Ha-, and N-*ras*) are small GTPase proteins that relay signals from the outside of the cell into the cell's nucleus. Certain missense point mutations, particularly those in codons 12 and 61 (such as amino acid valine replacing amino acid glycine in codon 12), obliterate GTPase activity and lead to constitutively active Ras protein, found in many cancers. Here, we used NIH3T3 fibroblasts transformed by Ha-*RAS*^{Val12} oncogene as a model.

9.2 Effects of JNK inhibitors and dominant negative mutants of SEK1/MKK4 and JNK1 on cell transformation

JNK and ERK pathways are usually involved in c-Jun activation. We had previously found a constitutive activation of JNK and phosphorylation of c-Jun at serines 63 and 73 in ODC-transformed cells (Hölttä et al., 1998). Here, we examined the significance of JNK activation in ODC-, AdoMetDC-, and RAS-transformed cells by using two different inhibitors of JNK, L-JNK inhibitor 1 (Dickens et al., 1997) and JNK inhibitor II (SP600125) (Bennett et al., 2001;reviewed in Koch et al., 2015) as well as dominant negative mutants of JNK1 and its upstream activator MKK4. L-JNK inhibitor 1 blocks the activities of JNK1 and JNK2, and JNK inhibitor II blocks the activities of all three JNKs. Both of these inhibitors were able to reverse the morphology of Odc cells, but only partially. When using dominant negative mutants of SEK1/MKK4 in Odc and Amdc cells, the transformed morphology was also partially reversed. The same was seen after using dominant negative mutant of JNK1 in both cell lines. This shows that the MKK4/JNK pathway is involved in ODC- and AdoMetDC- induced transformations, but may not be the only one. We did not find ERK or p38 pathways to be activated (data not shown), but other pathways may be involved. DN SEK1 and DN JNK1 had no effect in Amdc-as cells supplemented with spermidine.

9.3 Effects of dominant negative mutants of c-Jun on cell transformation

To examine the significance of c-Jun phosphorylation and activation in ODC- and *ras*- induced transformations, we used two phosphodeficient mutants of c-jun: c-Jun^{S63,73A} and c-Jun^{S63,73A,T91,93A}, and TAM67. In TAM67, most of the transactivation domain (amino acids 3-122) of c-Jun is deleted (Brown et al., 1993). Schematic maps of these dominant negative mutants of c-Jun are shown in Figure 7.

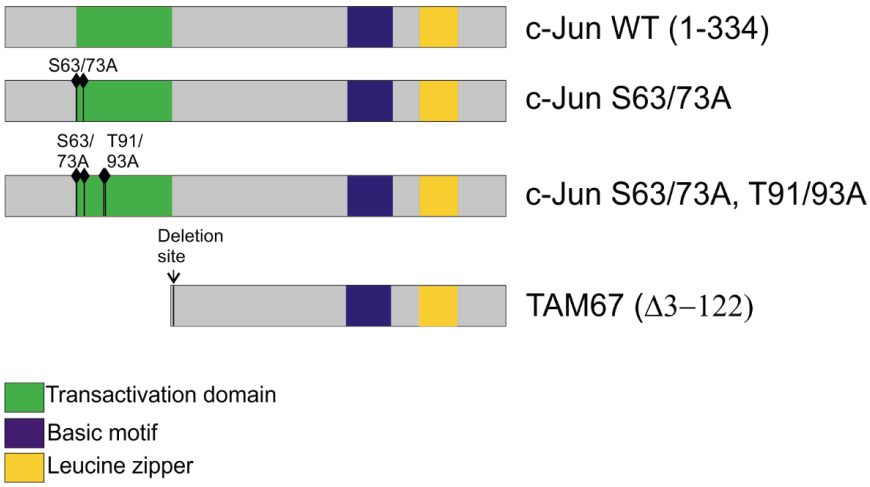
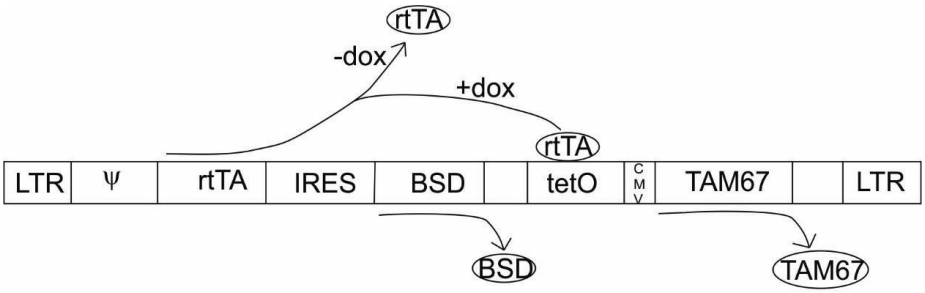


Figure 7. Schematic structures of c-Jun and different dominant negative mutants of c-Jun.

TAM67 can inhibit AP-1 activity through at least two mechanisms: blocking and quenching (Brown et al., 1996; Brown et al., 1994). In blocking, TAM67 homodimer binds DNA, blocking the response element, and in quenching, TAM67 heterodimer binds DNA but does not activate transcription. TAM67 was transfected to AdoMetDC sense- and antisense-induced transformed cell lines Amdc and Amdc-as. Previously, TAM67 has been used to investigate the role of AP-1 in other cell types as well (Li, J. J. et al., 2000; Liu, Y. et al., 2002). We found phosphorylation of c-Jun to be important for the transformed phenotype of Amdc, Odc, and E4 cells, but TAM67 had the greatest effect on the morphology, reversing the transformed phenotype of all cells completely. We further generated cell lines carrying a tetracycline-inducible expression system of the TAM67 (pLRT-TAM67). Figure 8 presents a diagram of the reverse tet-regulated retroviral vector construct, where the expression of TAM67 can be induced by doxycycline. After transfection of the cells with the construct, numerous cell clones with altered morphology were isolated and the expression levels of TAM67 (without or with doxycycline induction) analyzed by Western blotting. The best clones with a minimal leakage and strong TAM67 expression after induction were then selected for further studies. TAM67 expression was also found to downregulate the endogenous c-Jun expression, likely due to its binding to a variant c-Jun response element in the promoter region of the c-Jun gene itself. Most interestingly, the transformed morphology of the cells could be reversibly regulated by pLRT-TAM67 expression. In the presence of doxycycline, the transformed morphology of the

cells was reversed to a normal flattened phenotype within 1-3 days, and after removal of doxycycline the cells again returned to the transformed state.



LTR: long terminal repeat of Moloney murine leukemia virus; ψ packaging signal; rtTA: the reverse tetracycline controlled transactivator; IRES: internal ribosome entry site from the encephalomyocarditis virus; BSD: the blasticidin S deaminase; tetO: a heptamerized tet operator sequence; CMV: minimal human cytomegalovirus immediate early promoter; TAM67: transactivation domain deletion mutant of c-Jun

Figure 8. Schematic model of the reverse tet-regulated retroviral vector, where the expression of TAM67 can be induced by doxycycline (modified from (Watsuji et al., 1997). The addition of doxycycline leads to binding of rtTA to tetO, which allows the activation of TAM67. Blasticidin is used in the selection of the cells with the vector.

9.4 TAM67 INHIBITS COLONY FORMATION OF ADOMETDC- AND ODC-TRANSFORMED CELLS IN SOFT AGAR, THEIR INVASION IN 3D-MATRIGEL, AND PROLIFERATION *IN VITRO*

In Amdc and Amdc-as+spd cells, the expression of TAM67 inhibited the growth of the cells in soft agar (I). The same was true for the ODC-transformed Odc-pLRT-TAM67 cells, after induction of TAM67 expression by doxycycline (II). TAM67 further effectively inhibited the invasion of ODC- and AdoMetDC-transformed cells as well as RAS-transformed E4 cells in 3D-Matrigel (II, III). In addition, the inducible expression of TAM67 was found to inhibit the proliferation of ODC-, AdoMetDC-, and c-Ha-*ras*^{val12}-transformed cells (II, and data not shown). The effects of TAM67 were confirmed not to be due to TAM67 interfering with the AdoMetDC or ODC activities.

9.5 TAM67 INHIBITS TUMOR FORMATION IN *NUDE* MICE

To test the tumorigenicity of the ODC-transformed cells in detail, Odc-pLRT-TAM67 cells were inoculated subcutaneously at both flanks of the *nude* mice. The mice with induced TAM67 expression developed only very small or no tumors during the treatment period of 17

days, while the controls (uninduced cells) developed large tumors (II). In line with our study, TAM67 has also been shown to have antitumor effects in other mouse xenograft models of cancer such as those of colon cancer and non-small cell lung cancer (Suto et al., 2004; Shimizu et al., 2008).

10. IDENTIFICATION OF C-JUN-REGULATED AND TRANSFORMATION-ASSOCIATED GENES BY MICROARRAY ANALYSES (III, IV)

We next examined the transformation-associated gene expression changes regulated by c-Jun in ODC- and AdoMetDC-transformed cells and inducible Odc-, Amdc-, and Amdc-as-pLRT-TAM67 cell lines by Incyte Genomics cDNA and Affymetrix's oligonucleotide microarrays (III, IV). Relatively few genes were found to be involved in the transformations by the cDNA arrays (see Tables 9-14), including upregulated cyclin D1, proliferin, high mobility group protein, prothymosin β4, integrin β7, and integrin α6, and downregulated cysteine-rich 61 (CYR61), fibrillin-1, fibulin-5, and lysyl oxidase. These genes were confirmed to be differentially expressed by Affymetrix's microarray analyses (III, IV and (Nummela et al., 2006).

Table 9. Most downregulated genes in Odc-pLRT-TAM67 cells following TAM67 expression, as analyzed with Incyte Genomics Mouse UniGene1 cDNA LifeArrays.

Genbank	Gene	Gene description	Odc TAM67 +dox vs. -dox*
AI152562	*Prl2c5*	Prolactin family 2, subfamily c, member 5	-2.4
AA066647	*Lgals3*	Lectin, galactose binding, soluble 3	-2.3
AA067083	*Hmga1-rs1*	High mobility group AT-hook I, related sequence 1	-2.2
AA870247	*Prl2c2*	Prolactin family 2, subfamily c, member 2	-2.1
AA671769	***Itgb7***	**Integrin beta 7**	-2.0
AI466833	*Ngef*	Neuronal guanine nucleotide exchange factor	-1.8
AA185547	*Kcnn4*	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	-1.8
AI225491	*Fosl1*	Fos-like antigen 1	-1.8
AA619763	*Ctsl*	Cathepsin L	-1.6
AI120968	*Sgk1*	Serum/glucocorticoid regulated kinase 1	-1.5
AA066601	*Wfdc 15b*	WAP four-disulfide core domain 15B	-1.5
AA760223	*Emp3*	Epithelial membrane protein 3	-1.5
W14224	*Ndrg1*	N-myc downstream regulated gene 1	-1.5
AA088968	***Itga6***	**Integrin alpha 6**	-1.5
AA117547	*Ccnd1*	Cyclin D1	-1.5

*Expression changes of Odc-pLRT-TAM67 cells between cells with 1 μg/ml doxycycline (+dox) and without doxycycline (-dox) after three days' induction. The values are fold changes.

Table 10. Most upregulated genes in Odc-pLRT-TAM67 cells following TAM67 expression, as analyzed with Incyte Genomics Mouse Unigene1 cDNA LifeArrays.

Genbank	Gene	Gene description	Odc TAM67 +dox vs. –dox*
AA437518	*Fbln5*	Fibulin-5	4.4
AA273494	*Vdr*	Vitamin D receptor	3.5
AA221794	*Rgs2*	Regulation of G-protein signaling 2	3.1
AA711852	*Adamts1*	A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 1	2.9
AI020539	*Slpi*	Secretory leukocyte peptidase inhibitor	2.8
W11432	*Amotl2*	Angiomotin-like 2	2.7
W83882	***Lox***	**Lysyl oxidase**	2.5
AA733629	*C3*	Complement component 3	2.4
AA267178	*Cyp1b1*	Cytochrome P450, family 1, subfamily b, polypeptide 1	2.1
AA037995	*Mfap5*	Microfibrillar associated protein 5	2.1

*Expression changes of Odc-pLRT-TAM67 cells between cells with 1 μg/ml doxycycline (+dox) and without doxycycline (-dox) after three days' induction. The values are fold changes.

Table 11. Most downregulated genes in Amdc-pLRT-TAM67 cells following TAM67 expression, as analyzed with Incyte Genomics Mouse Unigene1 cDNA LifeArrays.

Genbank	Gene	Gene description	Amdc TAM67 +dox vs. –dox*	Amdc TAM67 +dox vs. –dox**
AI152562	*Prl2c5*	Prolactin family 2, subfamily c, member 5	-2.9	-5.1
AI466833	*Ngef*	Neuronal guanine nucleotide exchange factor	-2.6	-6.6
AA420078	*Itgbl1*	Integrin, beta-like 1	-2.4	-2.9
AA067083	*Hmga1-rs1*	High mobility group AT-hook I, related sequence 1	-2.4	-3.0
AA124623	*Evi2*	Ecotropic viral integration site 2	-2.3	-4.0
AA185547	*Kcnn4*	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	-2.2	-3.9
AA870247	*Prl2c2*	Prolactin family 2, subfamily c, member 2	-2.0	-4.3
AA117547	*Ccnd1*	Cyclin D1	-2.0	-2.9
AA667906	*Csrp2*	Cysteine and glycine-rich protein 2	-2.0	-3.4
AA798948	*Cav1*	Caveolin 1, caveolae protein	-1.9	-2.2
AA671769	***Itgb7***	**Integrin beta 7**	-1.9	-3.7
W09641	*Tmsb4x*	Thymosin, beta 4, X chromosome	-1.8	-3.1
AA619763	*Ctsl*	Cathepsin L	-1.7	-2.7
AA088968	***Itga6***	**Integrin alpha 6**	-1.5	-2.8

Expression changes of Amdc-pLRT-TAM67 cells between cells with 1 μg/ml doxycycline (+dox) and without doxycycline (-dox) after *three days' induction and after **six days' induction. The values are fold changes.

Table 12 Most upregulated genes in Amdc-pLRT-TAM67 cells following TAM67 expression, as analyzed with Incyte Genomics Mouse Unigene1 cDNA LifeArrays.

Genbank	Gene	Gene description	Amdc TAM67 +dox vs. -dox*	Amdc TAM67 +dox vs. –dox**
AA510298	*Plau*	Plasminogen activator, urokinase	3.2	3.7
W12942	*Tnc*	Tenascin C	3.2	3.2
AA437518	*Fbln5*	Fibulin-5	2.9	4.6
W16059	*Gsto1*	Glutatione S-transferase omega 1	2.8	<1.5
AW210317	*Fbn1*	Fibrillin 1	2.7	4.1
AA209882	*Nr4a1*	Nuclear receptor subfamily 4, group A, member 1	2.6	2.1
AI020539	*Slpi*	Secretory leukocyte peptidase inhibitor	2.5	3
AA466852	*Cyr61*	Cysteine rich protein 61	2.4	4.7
AI155437	*Akr1c18*	Aldo-keto reductase family 1, member C18	2.4	5
AI892243	*Fth1*	Ferritin heavy chain 1	2.4	2.4
AA562569	*Npc2*	Niemann Pick type C2	2.3	2
AA656268	*Pde4b*	Phosphodiesterase 4B, cAMP-specific	2.3	2.3
AA510727	*Pla2g4a*	Phospholipase A2, group IVA	2.2	1.6
AA547214	*Irf9*	Interferon regulatory factor 9	2.1	1.6
AA250679	*Akr1c12*	Aldo-keto reductase family 1, member C12	2.1	3.1
W88093	*Mgp*	Matrix gamma-carboxyglutamate (gla) protein	2.1	3.3
AA855996	*Bgn*	Biglycan	2.1	2.5
AI447967	*Maml2*	Mastermind like 2 (Drosophila)	2.1	<1.5
AA197601	*Mgat4a*	Mannoside acetylglucosaminyltransferase 4, isoenzyme A	2.1	1.8
AI019469	*Glul*	Glutamate-ammonia ligase (glutamine synthetase)	2.1	3.1
W13004	*Ptgis*	Prostaglandin I2 (prostacyclin) synthase	2.1	2.2
AA037995	*Mfap5*	Microfibrillar-associated protein 5	<1.5	2.3
W83882	***Lox***	**Lysyl oxidase**	<1.5	2.1

Expression changes of Amdc-pLRT-TAM67 cells between cells with 1 μg/ml doxycycline (+dox) and without doxycycline (-dox) after *three days' induction and after **six days' induction. The values are fold changes.

Table 13. Most downregulated genes in Amdc-as-pLRT-TAM67 cells following TAM67 expression, as analyzed with Incyte Genomics Mouse Unigene1 cDNA LifeArrays.

Genbank	Gene	Gene description	Amdc-as TAM67 +dox vs. –dox*
AA067083	*Hmga1-rs1*	High mobility group AT-hook I, related sequence 1	-2.7
AA771678	*Prkg2*	Protein kinase, cGMP-dependent, type II	-2.5
AI152562	*Prl2c5*	Prolactin family 2, subfamily c, member 5	-2.4
AI466833	*Ngef*	Neuronal guanine nucleotide exchange factor	-2.3
W15804	*Igfbp6*	Insulin-like growth factor binding protein 6	-2.2
AI120968	*Sgk1*	Serum/glucocorticoid regulated kinase 1	-2.2
AA185547	*Kcnn4*	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	-2.1
AI225491	*Fosl1*	Fos-like antigen 1	-2.0
AA671769	*Itgb7*	Integrin beta 7	-2.0

*Expression changes of Amdc-as-pLRT-TAM67 cells between cells with 1 μg/ml doxycycline (+dox) and without doxycycline (-dox) after three days' induction. The values are fold changes.

Table 14. Most upregulated genes in Amdc-as-pLRT-TAM67 cells when TAM67 expression is induced by doxycycline, as analyzed with Incyte Genomics Mouse Unigene1 cDNA LifeArrays.

Genbank	Gene	Gene description	Amdc-as TAM67 +dox vs. –dox*
AA711852	*Adamts1*	A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif 1	3.9
AA037995	*Mfap5*	Microfibrillar-associated protein 5	3.5
AA755007	*Dcn*	Decorin	3.4
AA733629	*C3*	Complement component 3	3.3
AI047160	*Ptn*	Pleiotrophin	2.8
AA437518	*Fbln5*	Fibulin-5	2.8
AI019567	*Tlr1*	Toll-like receptor 1	2.7
AA444232	*Akap12*	A kinase (PRKA) anchor protein (gravin) 12	2.6
AA796641	*Clec3b*	C-type lectin domain family 3, member 3	2.6
AI893922	*Ptx3*	Pentaxin-related gene	2.5
W71229	*Adamts2*	A disintegrin-like and metallopeptidase (reprolysin type) with thrombosponding type 1 motif 2	2.4
AI604159	*Fzd1*	Frizzled homolog 1 (Drosophila)	2.3
AA543149	*Il6st*	Interleukin 6 signal transducer	2.2
AA874687	*Fyn*	Fyn proto-oncogene	2.2
AA221794	*Rgs2*	Regulator of G-protein signaling 2	2.1
AA518187	*Ogn*	Osteoglycin	2.1
AA475186	*Irs1*	Insulin receptor substrate 1	2.0
W53231	*Cd82*	CD82 antigen	2.0
AA671168	*Anxa6*	Annexin A6	2.0
AI020539	*Slpi*	Secretory leukocyte peptidase inhibitor	2.0
W83882	***Lox***	**Lysyl oxidase**	1.5

*Expression changes of Amdc-as-pLRT-TAM67 cells between cells with 1 μg/ml doxycycline (+dox) and without doxycycline (-dox) after three days' induction. The values are fold changes.

Previously, the cell cycle protein cyclin D1 has been reported to be a target gene of c-Jun. Cyclin D1 functions as a regulator of CDK4 and CDK6 kinases, whose activity is required for cell cycle G1/S transition. Hennigan and Stambrook (2001) found that a high level of TAM67 expression inactivates the cyclin D1:CDK4/6 complex and thus arrests fibrosarcoma cells in G1. Proliferin (also known as mitogen-regulated protein) is involved in angiogenesis (Toft et al., 2001) and AP-1 has been found to be the mediator of serum-dependent proliferin expression in mouse fibroblasts (Groskopf and Linzer, 1994). Hommura et al. (2004), in turn, have reported that high mobility group protein AT-hook 1 (HMGA1 or HMG-I/Y) is a direct transcriptional target of c-Jun and is necessary for c-Jun-induced anchorage-independent growth in Rat1a cells. Dhar *et al.* (2004) showed HMGA1 to be a TAM67 target gene and related to transformation. HMGA1 binds to the minor groove of chromosomal DNA and regulates gene expression. In pancreatic adenocarcinoma, HMGA1 has further been suggested

to be a possible determinant of invasiveness and metastasis (Liau et al., 2006). In addition, we have previously reported the actin sequestering protein thymosin β4 to be upregulated in Amdc cells and to be regulated by c-Jun (Nummela et al., 2006). This regulation was, however, probably indirect because the decrease in thymosin β4 mRNA following TAM67 induction was a relatively slow process. The genes found to be downregulated in transformed cells and upregulated after TAM67 induction were mostly extracellular matrix proteins. For example, CYR61 (CCN1) is a secreted matricellular protein that is a mediator of collagen homeostasis and known to be regulated by AP-1 (Quan et al., 2010). FBLN5 is an integrin-binding extracellular matrix protein that is important for embryonic development and organogenesis (Noda et al., 2015). It acts as an elastogenic organizer by depositing on microfibrils, promoting aggregation of tropoelastin molecules through coacervation, and interacting with the elastin crosslinking enzymes LOXL1, 2, and 4 (Hirai et al., 2007). Indeed, microfibrils, such as fibrillin 1, act as scaffolds in the morphogenesis of elastic fibers. FBLN5 is further a multifunctional signaling molecule, which, in addition to its structural function, mediates cell-cell and cell-matrix communication (Albig and Schiemann, 2005). FBLN5 is known to stimulate MAPKs that enhance AP-1 activity stimulated by TGF-β. In tumorigenesis, FBLN5 is mainly a tumor suppressor through its control of cell proliferation, motility, and angiogenic sprouting (Albig and Schiemann, 2005). Surprisingly, it has also been shown that FBLN5 stimulates DNA synthesis and motility in fibroblasts and fibrosarcoma cells (Schiemann et al., 2002). In our study, FBLN5 was downregulated in transformed cells in a c-Jun regulatable manner. However, when Odc and Amdc cells were compared with their normal counterparts, no difference was detected in FBLN5 expression. Previous findings suggest that the downregulated expression of *FBLN5* may enable cancer formation and progression, as it functions as a tumor suppressor by inhibiting migration and invasion of ovarian cancer cells (Heo et al., 2015), cell proliferation and invasion of gliomas (Sheng et al., 2015) and metastasis of lung cancer (Chen et al., 2015). Microfibrillar-associated protein 5 (MFAP5) is also a multifunctional protein playing a role in cell signaling during microfibril assembly (Lemaire et al., 2007), elastinogenesis, and cell survival. However, its expression has mainly been reported to increase in human cancers (Leung et al., 2014).

Of the detected gene expression changes, we further examined integrins α6 and β7 because they were found to be both upregulated in transformed cells compared with parental cells and downregulated in transformed cells after TAM67 induction. We also chose lysyl oxidase for

additional studies since it was both downregulated in transformed cells compared with normal cells and upregulated in transformed cells after TAM67 induction.

11. FUNCTIONAL CHARACTERIZATION OF C-JUN-REGULATED AND TRANSFORMATION-ASSOCIATED GENES

11.1 INTEGRINS IN MOUSE FIBROSARCOMA CELLS (III)

Integrins are a family of cell surface transmembrane glycoproteins that function primarily as receptors for ECM ligands, including secreted ECM proteins fibronectin, laminin, and collagen. Integrins are composed of non-covalently associated α and β subunits forming heterodimers with a large extracellular domain that binds ECM molecules and a short cytoplasmic tail (an exception is the β4 subunit) that links ECM to the actin cytoskeleton (reviewed in Hynes, 2002; Luo et al., 2007; Springer and Wang, 2004; Kadry and Calderwood, 2020). Altogether 18 α and 8 β subunits exist that combine into at least 24 different heterodimers (reviewed in Takada et al., 2007) exhibiting overlapping but non-redundant functions (reviewed in Hynes, 2002; Luo et al., 2007). Integrins mediate interactions between cells and their extracellular microenvironment. In the "inside-out" signaling, integrin activation is triggered by a cytoplasmic signal or changes in the cytoskeleton, which lead to the modulation of integrin affinity for extracellular ligands. In addition to ECM components as ligands, some integrins bind to counter-receptors on other cells. The "outside-in" signaling, in turn, activates a number of signaling pathways that are important in the regulation of survival, proliferation, gene transcription, differentiation, migration, and apoptosis (reviewed in Hynes, 2002; Luo et al., 2007; Takada et al., 2007; Bianconi et al., 2016). Integrins can further regulate cell shape and stress fiber formation by activating kinases that phosphorylate cytoskeletal proteins (reviewed in Romero et al., 2020; Bianconi et al., 2016).

When the extracellular environment of the cancer cell changes, for example, upon its proteolytical degradation or stiffening, integrins sense these changes and trigger a range of cellular responses, but also play a crucial role in promoting a more malignant cancer cell phenotype. Indeed, integrins have an important role in different steps of cancer progression, including uncontrolled and limitless proliferation, cell survival and evasion of apoptosis, detachment, intravasation into lymphatic and blood vessels, migration and invasion of tumor cells, metastasis, and promotion of tumor angiogenesis (reviewed in Sun et al., 2014; Seguin et al., 2015; Ganguly et al., 2013; Huttenlocher and Horwitz, 2011; Garmy-Susini and Varner, 2008; Bianconi et al., 2016; Onodera et al., 2013; Arias-Mejias et al., 2020).

11.1.1 Integrins α6 and β7 are upregulated in AdoMetDC-transformed fibroblasts in a c-Jun-regulatable manner

Integrins α6 and β7 were found to be overexpressed in Amdc cells and downregulated in normalized Amdc-pLRT-TAM67 cells after TAM67 induction. Thus, these gene expression changes were both c-Jun- and transformation-dependent. As integrin β7 is thought to be leukocyte-specific, its overexpression in fibrosarcoma cells is an interesting finding. Previously, integrin β7 has been identified as a target gene of the oncogene c-MAF, which belongs to the AP-1 family of transcription factors (Hurt et al., 2004). The overexpression of integrins α6 and β7 was verified at the RNA level by RT-PCR and at the protein level by immunofluorescent staining of 4N and Amdc cells, and Amdc-pLRT-TAM67 cells with and without doxycycline. The expression of integrin α6 at protein level was additionally confirmed with FACS analysis and Western blotting.

Integrin β7 overexpression has also been detected in multiple myeloma cells, where it mediates regulation of adhesion, migration, and invasion (Neri et al., 2011) and in different lymphomas, including mantle cell lymphoma, thymic lymphoma, and mucosa-associated T- and B-cell non-Hodgkin lymphomas (Morito et al., 2006). Notably, the integrin β7 inducer c-MAF is overexpressed in nearly 50% of multiple myeloma patients (Hurt et al., 2004). Integrin α6, in turn, has been found to be overexpressed in esophageal squamous cell carcinomas and in hepatocellular carcinoma cells, where it was suggested to promote proliferation, invasion, and metastasis of the cells (Kwon et al., 2013; Ma et al., 2017; Lv et al., 2013).

11.1.2 Integrin β1 is a dimerization partner of integrin α6 in Amdc cells

The integrin subunit α6 can form heterodimers with integrin subunits β1 or β4, the ligand of both integrins α6β1 and α6β4 being laminin. The main dimerization partner of integrin α6 in Amdc cells was explored by immunoprecipitation analysis utilizing two different Itgα6 antibodies. The captured proteins in the Itgα6 immunoprecipitates were immunoblotted with antibodies specific to β1 and β4. In addition, the expression levels of these integrin subunits were analyzed by RT-PCR and Affymetrix microarrays. In these analyses, integrin β4 subunit was not found to be complexed with α6 subunit at all or only minimally, whereas α6β1 was detected as the major heterodimer expressed in Amdc cells. Integrin β1 is a more universal integrin subunit than β4, with most cells in the body expressing it. In addition, it can form altogether 11 different integrin heterodimers in all four receptor groups [laminin, RGD (fibronectin, vitronectin, and fibrinogen), collagen, and leukocyte-specific receptors (reviewed

in (Barczyk et al., 2010)]. Integrin subunit β4, in turn, can form a heterodimer only with α6. Nevertheless, integrin α6β4 has been found to be upregulated in many tumor types, being also a potential target for cancer therapy (reviewed in Giancotti, 2007).

Integrin subunit β7 heterodimerizes with integrin subunits α4 and αE. The ligands of α4β7 are fibronectin, vascular cell adhesion molecule 1 (VCAM-1), and mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1), which are present on the surface of endothelial cells, bone marrow stromal cells, and epithelial cells. On the other hand, αEβ7 retains intraepithelial lymphocytes within the gut epithelium by binding E-cadherin (Neri et al., 2011). However, we were unable to clarify the main dimerization partner of Itgβ7 in Amdc cells by immunoprecipitation. Yet, our initial results by RT-PCR showed that Amdc cells express Itgαe. It remains to be elucidated which of the integrins, α4β7 or αEβ7, is present in fibrosarcoma cells.

11.1.3 Integrin α6β1 is involved in adhesion of Amdc cells to laminin

Because integrin α6β1 is a laminin receptor, we used function-blocking antibodies against integrin α6 and its partner, integrin β1, with Amdc cells and tested their ability to inhibit binding to laminin and Matrigel, which consists mostly of laminin. The function-blocking antibody of integrin subunit α6 considerably inhibited the adhesion to laminin, and the integrin subunit β1 antibody inhibited the binding completely, probably because integrin β1 subunit has numerous dimerization partners. However, the function-blocking antibody of integrin subunit β7 did not inhibit Amdc cells from binding to fibronectin, the ligand of integrin α4β7.

11.1.4 Integrin α6β1 is involved in invasion

As Amdc cells are highly invasive, we further examined whether the upregulation of integrin α6 is necessary for invasion. In these assays, we utilized again function-blocking integrin α6 antibodies (as well as neutralizing antibodies for its partner Itgβ1) and 3D Matrigel matrix. Indeed, both function-blocking integrin α6 and integrin β1 antibody fully inhibited invasion. Notably, in addition to the Amdc cells, also human HT-1080 fibrosarcoma cells were found to rely on integrin α6β1 to be able to invade. Integrin β7 antibody, in turn, had no effect on invasion. The functional significance of integrin β7 in transformation remains to be clarified in fibrosarcoma cells.

11.1.5 Integrin α6 is found in invasion fronts of human high-grade fibrosarcomas

The potential clinical significance of integrin α6 in human fibrosarcomas and other soft-tissue sarcomas was then studied by immunohistochemistry. Interestingly, strong staining of Itgα6 was detected in the invasion fronts of high-grade tumors. Moreover, integrin α6 immunoreactivity was correlated with the expression of activated c-Jun.

11.2 LYSYL OXIDASE FAMILY PROTEINS IN FIBROSARCOMA AND MELANOMA CELLS (IV)

The lysyl oxidase family consists of lysyl oxidase (LOX) and lysyl oxidase-like 1-4 (LOXL1, LOXL2, LOXL3, and LOXL4) proteins. They are copper-dependent amine oxidases containing differing N-terminal regions and a conserved C-terminal domain (reviewed in Finney et al., 2014). The structures of LOX family proteins are shown in Figure 9. The C-terminal region contains a copper-binding motif for the protein conformation and a lysyl-tyrosyl-quinone (LTQ) cofactor for the amine oxidase activity needed in crosslinking of collagen and elastin (reviewed in Nishioka et al., 2012). The N-terminal domains are more divergent, conferring individual functions (Molnar et al., 2003): LOX and LOXL1 contain prosequences enabling their secretion as inactive proenzymes and directing them to interact with the ECM. LOXL2, LOXL3, and LOXL4, in turn, contain four scavenger receptor cysteine-rich domains, which are thought to be involved in cell adhesion and protein-protein interactions.

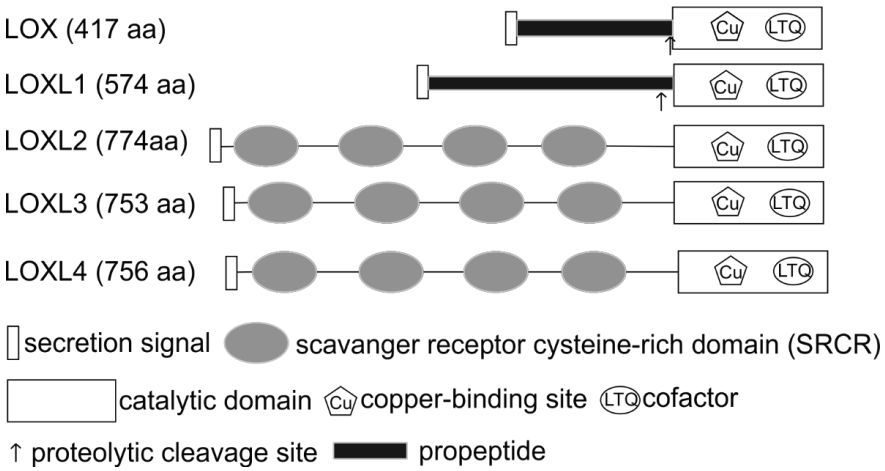


Figure 9. Structure of lysyl oxidase (LOX) and the other lysyl oxidase family members, lysyl oxidase-like 1-4 (LOXL 1-4). Modified from reviews of Finney et al. (2014), Nishioka et al. (2012), and Cox and Erler (2013).

LOX is synthesized and secreted as a 50 kDa glycosylated proenzyme (Pro-LOX), which is cleaved extracellularly by bone morphogenetic protein 1 (BMP-1) and related proteases (tolloid-like 1 and 2) to the functional 32 kDa enzyme (LOX) and an 18 kDa propeptide (LOX-PP) (Uzel et al., 2001). The activation of LOX is dependent on its binding to the fibronectin matrix (Fogelgren et al., 2005) and periostin, which binds both to BMP-1 and fibronectin (Maruhashi et al., 2010). LOX plays an essential role in the crosslinking of collagen and elastin in the ECM (Kagan and Li, 2003). In addition to having a role in connective tissue homeostasis, LOX is involved in the regulation of various cellular processes, such as cell adhesion, invasion, and migration, and also cell polarity and epithelial-mesenchymal transition in hypoxic conditions (El-Haibi et al., 2012; Kasashima et al., 2015; reviewed in Csiszar, 2001; Molnar et al., 2003). Further, it has been reported to have functions in the cytoplasm (Jansen and Csiszar, 2007) and nucleus (Li, W. et al., 1997; Nellaiappan et al., 2000), where it may regulate transcription (Okkelman et al., 2014).

LOX is essential for normal development, and the failure of LOX function can lead to several diseases (Tsuda et al., 2003). Indeed, deficient activity of LOX is related to many connective tissue disorders such as Ehler-Danlos syndrome, Menke's syndrome, and cutis laxa (Kuivaniemi et al., 1985; Khakoo et al., 1997). Elevated LOX activity, in turn, is associated with various fibrotic diseases, including atherosclerosis, scleroderma, and liver cirrhosis (Kagan et al., 1981; Chanoki et al., 1995; Murawaki et al., 1991). Most interestingly, aberrant expression of LOX is found in many cancer cells, and paradoxically, LOX has been found to function as both a tumor promotor (Lee, Y. S. et al., 2017) and a tumor suppressor (reviewed in Siddikuzzaman et al., 2011).

11.2.1 Lox is downregulated in ODC-transformed mouse fibroblasts in a c-Jun-dependent manner

We found Lox to be downregulated in ODC-transformed Odc cells (both at the mRNA and protein levels) compared with normal N1 cells, and upregulated again in Odc-pLRT-TAM67 cells upon reversal of transformation by TAM67 expression.

11.2.2 Expression of Lox family members Lox-like1 and Lox-like3 is downregulated in Odc cells

In addition to Lox, we examined the expression levels of the other Lox family members, *Lox-like 1-4*, in Odc and parental N1 cells by Affymetrix microarrays and verified the results by RT-PCR. We found that, in addition to *Lox*, *Loxl1* and *Loxl3* were also downregulated in Odc cells when compared with N1 fibroblasts. Of these Lox family members, the highest expression levels were seen with *Lox* in the Odc cells. In contrast, *Loxl4* was slightly upregulated in Odc cells, and *Loxl2* was not expressed at detectable levels in these cell lines.

11.2.3 Expression levels of Lox family members are downregulated in RAS-transformed fibroblasts

Initially, LOX was identified as a "*ras* recision gene" (rrg) due to its ability to revert RAS-mediated transformation of NIH 3T3 fibroblasts (Contente et al., 1990). Thereafter, rrg was identified as *Lox* and proposed to be a tumor suppressor (Contente et al., 1990; Kenyon et al., 1991). Indeed, the expression of LOX has been shown to be decreased in various cells transformed by *ras* or *ras*-dependent oncogenes (Contente et al., 1990; Giampuzzi et al., 2001). Jeay *et al.* (2003) have further reported that the tumor suppressor action of LOX prevents the activation of NF-κB in the *ras*-mediated transformation.

In our study, we explored *Lox* in more detail and also *Lox-like 1-4* in HRAS-oncogene-transformed E4 cells. We found *Lox* expression to be extensively downregulated in E4 cells compared with normal N1 cells, but also the expression levels of *Loxl1*, *Loxl3*, and *Loxl4* were slightly reduced, as analyzed by Affymetrix microarray and RT-PCR analyses.

11.2.4 LOX family members are upregulated in melanoma cells

Previously, Nummela *et al.* (2012) had observed alterations in the expression levels of LOX family members, especially an upregulation of *LOXL2*, in melanoma cell lines compared with normal melanocytes by microarray analyses. Similarly, an upregulation of c-Jun expression and activity had been detected in melanomas (Spangler et al., 2011); reviewed in (Kappelmann et al., 2014). Furthermore, at least the *LOX* gene has been shown to have an AP-1/c-Jun binding site in its promoter region (Hamalainen et al., 1993; Papachroni et al., 2010). Consequently, we were interested in extending our studies to the expression levels of all LOX family members in different melanoma cell lines. The gene expression levels of *LOX* and the *LOX-like* family members were evaluated at the mRNA level using microarray and RT-PCR analyses. Variable expression patterns were observed, depending on the melanoma cell line. However, the expressions of *LOXL2* and *LOXL3* were upregulated in nearly all of the melanoma cell lines examined. The expression levels of all lysyl oxidase family members in melanocytes and melanoma cells, as analyzed by RT-PCR, are shown in Table 15 (see also IV, Figure 4). LOX and particularly LOXL2 have been often found to be overexpressed in other human cancers as well (reviewed in Barker et al., 2012; Moon et al., 2014; Wu, L. and Zhu, 2015).

Table 15. Expression of LOX family members in human melanocytes and melanoma cells.

Name	Description	LOX	LOXL1	LOXL2	LOXL3	LOXL4
42V	Primary melanocytes	-	-	-	-	-
MELA3	Primary melanocytes	-	-	-	-	-
MELA-TN45	Primary melanocytes	-	-	-	-	-
EL29	Primary melanoma	-	-	-	x	-
WM115	Primary melanoma cell line (vgp)	-	-	x	x	x
WM793	Primary melanoma cell line (vgp)	x	-	x	x	-
WM239	Metastatic melanoma cell line (lnm)	-	-	x	x	x
MM170	Metastatic melanoma cell line (lnm)	-	-	x	x	x
SK-MEL-28	Metastatic melanoma cell line (sm)	-	-	-	x	-
BLM	Metastatic melanoma cell line (lm)	x	x	x	-	-
SK-MEL-103	Metastatic melanoma cell line	-	-	x	x	-
SK-MEL-147	Metastatic melanoma cell line	x	x	x	x	-

vgp=vertical growth phase, lnm=lymph node metastasis, sm=skin metastasis, lm=lung metastasis

x=upregulated expression

Further, the protein expression levels of LOX and LOXL2 in human fibroblasts (HES) and three melanoma cell lines (SK-MEL-147, WM793, and WM239) were studied by Western blot analysis. The PRO-LOX protein level appeared to be high intracellularly, but it was found to be effectively processed to active LOX extracellularly in HES cells. In melanoma cells, the secreted protein levels of LOX were high only in WM793 cells, whereas the contents of LOXL2, both the cellular and secreted protein levels, were relatively high in all melanoma cell lines evaluated.

We found the mRNA expression levels of LOXL1 and LOXL2 genes to be markedly upregulated in the primary melanoma tissue specimens compared with human benign nevi. Most interestingly, high LOXL2 expression appeared also to be associated with the formation of metastases and shorter survival of patients ($p=0.004$).

11.2.5 LOX and LOX-like proteins are oppositely associated with invasion of ODC-transformed fibroblasts and melanoma cells

We then examined the functional significance of LOX in ODC-transformed fibroblasts and melanoma cells. Paradoxically, LOX was found to be downregulated in mouse fibrosarcoma cells and upregulated in human melanoma cells. Similarly, LOX has been reported to be downregulated in human basal and squamous cell carcinomas, gastric cancers, and osteosarcoma tissues (Bouez et al., 2006; Kaneda et al., 2004; Xu, X. et al., 2013), but overexpressed in human esophageal squamous cell carcinoma, lung adenocarcinoma, and colorectal cancer (Baker et al., 2011; Sakai et al., 2009; Wilgus et al., 2010). Further, LOX has been known to be inactivated by methylation and loss of heterozygosity in human gastric cancers (Kaneda et al., 2004).

To assess the role of LOX in Odc cells, we generated cell lines transfected with a tetracycline-inducible expression system of pro-LOX (Odc-pLRT-LOX). The best clones, with low background expression in the absence of doxycycline, were selected for further studies. First, we examined the cell proliferation rates and found that the induced expression of pro-LOX markedly inhibited the growth of Odc-pLRT-LOX cells in 2D culture. Next, we used a 3D Matrigel assay to mimic the *in vivo* situation and found that induced expression of LOX led to an inhibition of the invasion of Odc-pLRT-LOX cells as well. When the cells were further incubated with the LOX inhibitor BAPN in the absence or presence of doxycycline, there was no effect on cell invasion. BAPN is known to bind irreversibly to the LOX active site, blocking its activity, and thus, preventing LOX from catalyzing the conversion of lysine residues to reactive aldehydes and the formation of crosslinks (Tang et al., 1983; Tang et al., 1989). The structural formula of BAPN is shown in Figure 10. Importantly, our results show that the enzyme activity of LOX had no effect on the invasion of Odc-pLRT-LOX cells. Previously, Palamakumbura *et al.* (2004) have found that lysyl oxidase propeptide (LOX-PP) inhibits *Ras*-dependent cell transformation. Further, LOX-PP has been shown to inhibit the invasive phenotype and to function as a tumor suppressor in many transformed cell lines and cancers where the signals are mediated via RAS, e.g. in H1299 lung cancer and PANC-1 pancreatic cancer cell lines (Wu, M. et al., 2007) and epidermal growth factor receptor-2/neu-driven breast cancer cells (Min et al., 2007). Despite these findings, we did not observe any detectable increases in LOX-PP protein levels in the normal NIH3T3 compared with Odc cells or in Odc-pLRT-LOX cells upon doxycycline-induced expression of LOX and inhibition of cell proliferation and invasion. Our studies thus suggest that the inactive pro-LOX (together with LOX-PP) functions as a major tumor suppressor in Odc cells.

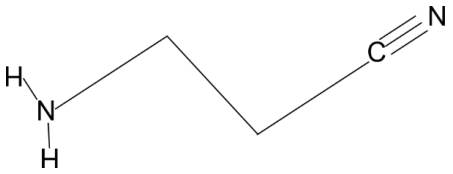


Figure 10. Structural formula of lysyl oxidase active site inhibitor β-aminopropionitrile (BAPN).

In addition to LOX, we investigated the significance of LOXL2 in melanoma cells. We tested the effects of BAPN on two melanoma cell lines, the primary melanoma cell line WM793 with low invasive capacity and the metastatic melanoma cell line SK-MEL-147 with high invasive activity, in 3D Matrigel. BAPN at a concentration of 500 μM effectively inhibited the invasion of WM793 cells, but the invasion of SK-MEL-147 cells was only slightly or moderately reduced. Our group previously found that the invasion of melanoma cells is promoted and guided by fibroblasts (Yin et al., 2012). Indeed, when co-culturing melanoma cells and fibroblasts in 3D Matrigel the invasion was increased, and the BAPN treatment led to a complete inhibition of the co-invasive growth of fibroblasts and melanoma cells. Cancer-associated fibroblasts (CAFs) have also been shown to play a crucial role in the promotion of cancer progression in many other cancer cells (Wen et al., 2019; Torres et al., 2015; Nguyen et al., 2019).

Finally, we examined the consequences of knocking down LOX and LOXL2 proteins in WM793 cells by specific shRNAs. Notably, both the capability of the cells to proliferate in 2D culture and the capacity of the cells to invade in 3D Matrigel were markedly reduced. The depletion of LOXL2 almost completely blocked the invasive growth capacity of the cells, even more effectively than BAPN, suggesting that not only the activity but also the protein content of LOXL2 is important in the regulation of proliferation and invasion of these cells.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

We first examined the significance of c-Jun phosphorylation and activation in AdoMetDC-, ODC-, and Ha-*ras*-transformed mouse fibroblast cell lines (Amdc, Odc, and E4 cells, respectively) by using dominant negative mutants of upstream kinases of the MAPK and JNK pathways, JNK inhibitors, phosphodeficient mutants of c-Jun, and transactivation domain deletion mutant of c-Jun (TAM67) and found them all to reverse the transformed phenotype to a variable extent. However, TAM67 was by far the most effective in reversing the transformed phenotype of these cells, indicating that phosphorylation is not the only way that c-Jun functions in transformation; other mechanisms are also involved. Further, we generated cell lines with a tetracycline-inducible expression system of TAM67. We were able to regulate the state of transformation and invasiveness of these cell lines, and, most importantly, also the tumorigenic activity of the cells in nude mice was blocked upon induction of TAM67 expression.

Next, these cell lines with inducible expression of TAM67 were used for screening the c-Jun-regulated and potentially transformation-specific gene expression changes by DNA microarray analyses. We identified several interesting gene expression changes, including the upregulation of integrin α6 (Itgα6) and integrin β7 (Itgβ7) and the downregulation of lysyl oxidase (Lox), in these fibrosarcoma cells. Functional analyses further revealed Itgα6 and Lox to be intimately involved in the regulation of invasion of these cells. The expression of Itgα6 was also found to be increased at the invasive edge of human high-grade fibrosarcomas. In the future, it will be interesting to examine the functional roles of c-Jun and integrin α6 in human fibrosarcomas in more detail. In addition, the behavior of the collagen and elastin crosslinking enzyme, LOX, as well as the other invasion-related proteins identified in the mouse fibrosarcoma cell lines, remains to be evaluated in human fibrosarcomas. Therapeutic strategies targeting these proteins, particularly the cell surface receptor integrin α6β1, may offer new means for treating aggressive fibrosarcomas.

Recent evidence has shown a double-edged role for LOX in transformation, functioning as both a tumor suppressor and a tumor promoter. Indeed, we also found that, in contrast to fibrosarcoma cells, LOX was upregulated in human melanoma cells. As one explanation for this conundrum, we found that it is the inactive, unprocessed LOX (pro-LOX) that functions as a tumor suppressor in the ODC- and RAS-transformed fibroblasts, and the cleaved, active

LOX that acts as a tumor promoter in human melanoma cells. In addition to LOX, we also observed LOXL2 to promote the proliferation and invasion of melanoma cells. It would be interesting to also investigate the functional significance of the other LOX-like family members, especially the often upregulated LOXL3, in melanoma cells. As LOXL2 was found to be upregulated in almost all melanoma cells evaluated and also in human melanomas, where high expression of LOXL2 was further associated with short survival of the patients, LOXL2 provides an attractive potential target for therapy in human melanomas. Moreover, since our study showed the involvement of c-Jun in the regulation of many critical genes in the transformation of fibrosarcoma cells, it would be interesting to more closely examine the role of c-Jun and its target genes in melanoma cells as well.

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Mari

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